INTRASPINAL TRANSPLANTATION OF MICROGLIA: STUDIES OF HOST CELLULAR RESPONSES AND EFFECTS ON NEURITIC GROWTH

By

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INTRASPINAL TRANSPLANTATION OF MICROGLIA: STUDIES OF HOST CELLULAR RESPONSES AND EFFECTS ON NEURITIC GROWTH

by

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Investigations into the cellular biology of microglial cells suggest these cells not only initiate the rejection of neural grafts into the central nervous system (CNS) but also secrete molecules that are potentially toxic to neurons. Studies of cytokine and growth factor production by activated microglia/brain macrophages, on the other hand, suggest they may directly or indirectly promote neuritic elongation in the injured CNS. In view of these contrasting perspectives, this dissertation utilizes a transplantation approach to investigate the influence on neuronal growth of tissue cultured microglia placed into the injured rat spinal cord. The specific focus is on the histological and immunohistochemical demonstration of neuritic ingrowth into various grafts containing matrices of gelfoam that were impregnated with cultured microglia or astrocytes.

Prominent neuritic ingrowth was observed in the microglia-impregnated implants that displayed intense OX-42-immunoreactivity (IR).

In contrast, astrocyte implants showed less neuritic growth-promoting effects. In addition to neuritic ingrowth into the microglia-seeded gelfoam implants, there was an infiltration of host cellular elements, many of which were laminin-IR and thus thought to be microvascular, Schwann cell and/or mesechymal infiltrates. Neuritic growth also was seen in activated microglial environments of the host outside the polymeric tube implants. Lastly, some control (i.e., cell-free) implants demonstrated a slight ingrowth of neurites, but this only occurred coincident with the infiltration of host cellular elements which consistently involved OX-42-IR microglia/brain macrophages.

Collectively, this evidence argues against a neurotoxic role played by microglia in the injured CNS. Accordingly, these cells may at least partially counteract the events that contribute to poor regeneration in the mammalian CNS. It remains to be determined, however, whether the neuritic growth-promoting effect observed is directly related to grafted microglia or secondarily associated with host cellular elements that could be more directly conducive to the outgrowth of neuronal processes.

CHAPTER 1
BACKGROUND

Spinal Cord Injury and Gliosis

After injury to the spinal cord there is an immediate extravasation of hemorrhagic elements into the lesioned area. This is followed by neutrophil invasion and neuronophagia (Means and Anderson, 1983). Macrophages and microglial cells accumulate at the lesion site during the first week. Astrocytic processes soon delineate an area of necrosis and form an intense astroglial lining that, in many instances, develops into a compact scar which can be invaded by Schwann cells (Dusart et al., 1992), as well as mesodermal elements that establish a dense collagenous matrix.

Astrocytes, together with other non-neuronal cellular elements (i.e,. fibroblasts), form densely interwoven scars that fill the space vacated by dead or dying cells that are thought to inhibit regeneration in the central nervous system (CNS) (Reier et al., 1983; 1988; Feringa et al., 1984; Bernstein et al., 1985; Liuzzi and Lasek, 1987). Studies characterizing successful axonal regrowth through scar tissue in the adult mammalian spinal cord demonstrate that neurites will invade traumatized tissue domains, but only in conjunction with nonneuronal elements, most notably ependymal and mononuclear cells (Guth et al., 1983; 1985). One mononuclear cell type endogenous to the CNS which becomes activated after CNS damage and whose effects on neuronal elongation and glial scar formation is poorly understood is the microglial cell.

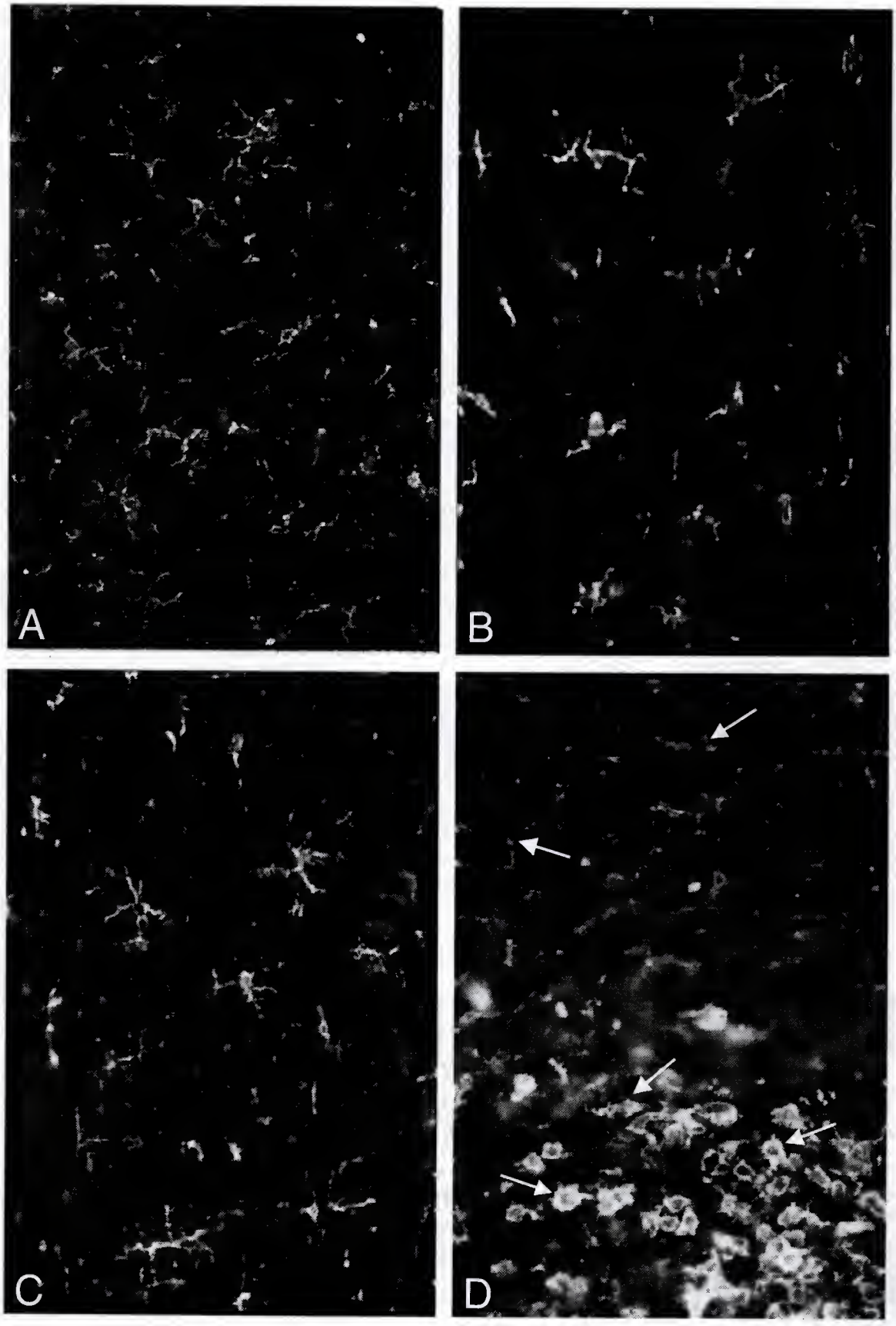
Overview of Microglial Ontogeny and Responses to Injury

During embryonic development, microglia have an ameboid morphology, are highly motile, and display phagocytic function thought to play a role in scavenging other dying cells during development (Ashwell, 1990). However, during the course of CNS development ameboid microglia disappear and ramified microglia, having a small soma with long branching processes, increase in number. It has been speculated that some of the ameboid microglia eventually differentiate into ramified microglia (Ling, 1981; Ling and Wong, 1993). The proposed origins of microglia, however, are still controversial. Whether they are ectodermally or mesodermally derived has been the focus of much debate (see Theele and Streit, 1993). Currently, the most plausible theory is that they initially derive from a precursor cell related to the monocyte/macrophage lineage associated with embryonic hemopoietic organs, such as the bone marrow, spleen, or liver.

Microglia have many phenotypic characteristics that vary depending on the stage of development, location within the CNS, and on whether the CNS has been perturbed by injury or disease. Studies also have demonstrated that microglia *in vitro* display polymorphic phenotypes that usually depend on the culturing conditions. This diverse morphology of microglia is thought to be closely associated with their functional state.

Ramified microglia in the normal, adult rat CNS are uniformly dispersed, unlike other glial cells, and are thought to be in a functional "resting state" (Figs. 1-1A-C). These glia represent endogenous cells of the CNS involved in ionic homeostasis and are thought to act as "pinocytotic filters" sampling the extracellular fluids for foreign antigens and other diffusible substances (Ward et al., 1991). When the CNS is injured or

Fig. 1-1. Photomicrographs displaying the heterogeneity of microglial phenotypes found within the rat spinal cord. All panels represent high magnifications of horizontal cryosections immunofluorescently stained with OX-42 antibody, recognizing the CR3 receptor expressed on microglia and macrophages (X320). A shows highly ramified, parenchymal microglia, B and C demonstrate the different microglial profiles found in white matter regions, D shows an injured area in the rat spinal cord that is highlighted by an increased hypertrophy of microglia and their transformation into brain macrophages. Note the polymorphic stages of activation (i.e., process-bearing to ameboid; arrows).


A
B
C
D

affected by disease, microglia become reactive and are thought to be functionally activated (Streit and Kreutzberg, 1988; Streit et al., 1988). Microglial activation involves cellular hypertrophy and retraction of cytoplasmic processes (Fig. 1-1D). In regions of necrosis, activated microglia can transform into brain macrophages that proliferate and phagocytose cellular debris (Streit and Kreutzberg, 1988; Graeber et al., 1989). Microglial activation also is characterized by marked changes in phenotype. This includes upregulation of existing surface antigens as well as *de novo* expression of such molecules (Graeber et al., 1988; Streit et al., 1989).

It is difficult to demonstrate conclusively the invasion of central nervous (CNS) tissue by blood monocytes because specific markers that distinguish macrophages from activated microglia do not exist. However, it appears that the increase in cell number after CNS damage is related to a recruitment and proliferation of microglial cells, as well as of blood monocytes (Schelper and Adrian, 1986; Graeber et al., 1989; Morshead and VaderKooy, 1990; Andersson et al., 1991; Marty et al., 1991). Andersson et al. (1992) report monocytic recruitment even in the absence of blood-brain barrier disruption. Mononuclear phagocytes clear cellular debris and secrete cytokines/growth factors which are involved in immunological responses to tissue repair. They also can express molecules that are potentially toxic to neural cells and may thus negatively affect the potential for regeneration.

Neurotoxicity and Regeneration-Inhibiting Effects of Microglia

In tissue culture, stimulation of microglia with lipopolysaccharide (LPS), interferon-gamma (IFN-γ), and/or zymosan A results in the elaboration of various noxious and cytotoxic agents (Banati et al., 1993), including reactive oxygen intermediates (Colton and Gilbert, 1987), tumor necrosis factor-alpha (TNF-α) (Frei et al., 1987; Sawada et al., 1989), glutamate

(Piani et al., 1991), and low molecular weight factors with neurotoxic activity (Giulian et al., 1993a,b). The progressive destruction of axotomized retinal ganglion cells has been shown to be enhanced or suppressed with macrophage-stimulating (MSF) or -inhibiting factors (MIF), respectively (Thanos et al., 1993). The retention of retinal ganglion cells by MIF treatment can lead to the regrowth of axons *in vivo* and *in vitro*. Although the mechanisms by which these factors produce their effects are poorly understood, the suggestion has been made that suppression of brain macrophages is essential for regeneration to occur.

Neurotrophic Properties of Microglia

In contrast to the proposed adverse nature of microglia/brain macrophages, it has been shown that changes in axonal growth-promoting properties of the injured CNS may be produced by mononuclear phagocytes that invade lesioned areas and then modify nonpermissive substrata (David et al., 1990). Microglia are known to secrete trophic molecules such as transforming growth factor-beta (TGF-β) (Constam et al., 1992; Lindholm et al., 1992; Finch et al., 1993; Kiefer et al., 1993), basic fibroblast growth factor (bFGF) (Shimojo et al., 1991), and nerve growth factor (NGF) (Mallat et al., 1989; Lindholm et al., 1992). This has prompted investigations into the putative neurotrophic properties of cultured microglia.

Studies in which neurons from various CNS regions have been co-cultured with purified microglia and/or microglia-conditioned medium concluded that both contact with microglia or growth in microglia-conditioned medium promoted neuronal survival and neuritic arborization (Nakajima et al., 1989,1993; Nagata et al., 1993a,b; Zhang and Federoff, 1993; Chamak et al., 1994). It remains to be determined, however, whether microglia produce similar effects in the injured CNS.

In Situ Grafting of Glia Derived from Tissue Culture

Over the years, neural tissue transplantation has served as an effective tool for investigating the nature of various cellular interactions in the injured CNS (Reier et al 1983; 1986; Houle and Reier, 1988; Jakeman and Reier, 1991). In this regard, glial cells from both the CNS and PNS have been transplanted into the CNS as whole (i.e., peripheral nerve) or dissociated tissue, as well as *in vitro*-derived cells. Using tissue culture as a source of donor cells allows the greatest control over transplant composition because such cells can be manipulated to some extent using specific culturing protocols and isolation techniques. Cultured oligodendrocytes transplanted into hypomyelinated rodents have been shown to form myelin sheaths with some myelination occurring at a distance from the transplantation site (Gout et al., 1988; Rosenbluth et al., 1990). Other groups have transplanted mouse glia to repopulate ethidium bromide lesions in rats and showed that even xenogeneic donor oligodendrocytes can remyelinate host axons (Crang and Blakemore, 1991).

Transplantation of astrocytes into the adult rat spinal cord results in the extensive migration of injected or graft-derived astrocytes (Goldberg and Bernstein, 1988; Bernstein and Goldberg, 1989; Wang et al., 1995). Cultured astrocytes have also been transplanted to examine their effects on remyelination (Franklin et al., 1991) and scar formation (Smith and Silver, 1988). Under certain circumstances, astroglia may provide a matrix that will support axonal growth *in vitro* and during axonal development and regrowth in mammalian CNS tissue (Matthews et al., 1979; David et al., 1984; Fishman and Kelly, 1984; Noble et al., 1984; Fallon, 1985; Guth et al., 1985; Miller et al., 1986; Smith et al., 1986; Assouline et al., 1987). Recently,

cultured astrocytes injected into hemisected adult rat spinal cords alone or presented in a gelfoam matrix were shown to reduce scarring, as measured by glial fibrillary acidic protein (GFAP) specific to astrocytes, and to increase the intensity of neurofilament staining (Wang et al., 1995). However, the prelabeled, grafted astrocytes migrated out of the Gelfoam implants as early as one week after implantation raising the possibilities that they either produced their effects early after grafting or that their emigration created a more permissive environment for neuritic growth.

Experimentally demyelinated CNS axons exert a powerful attractant and mitotic effect on transplanted Schwann cells (Blakemore, 1984; Crang and Blakemore, 1989). The invasion of myelinating Schwann cells into the CNS depends on the concurrent loss of both astrocytes and oligodendrocytes as demonstrated by the inability to detect Schwann cells after they are injected into tissue where astrocytes are present (Blakemore et al., 1986). The capacity of cultured Schwann cells to myelinate CNS axons in a glial deficient environment has been demonstrated when these cells were transplanted into ethidium bromide lesions in spinal cords irradiated with X-rays (Blakemore et al., 1987a,b). Enriched Schwann cell grafts also can reduce both post-traumatic cystic cavitation and astrocytic scar formation when injected into the site of an acute compression lesion (Martin et al., 1991).

Considerable axonal growth has been demonstrated through cultured, peripheral Schwann cells (combined with their collagen substratum) after placement into the aspiration cavities of adult rat spinal cords (Paino and Bunge, 1991; Paino et al., 1994). Recently, permselective, polymeric tubes were filled with cultured Schwann cells seeded in Matrigel™ (Collaborative Research, Inc.; Bedford, MA) to reconstruct complete fimbria-fornix and/or spinal cord resection cavities in rats (Hoffman and Aebischer, 1993; Xu et al.,

1995). These studies demonstrated both the regrowth of cholinergic axons, and the rostral/caudal ingrowth of spinal axons through the implants, respectively. It was concluded that Schwann-cell-seeded guidance channels provided directionally oriented biosynthetic bridges that induce CNS regeneration via a direct association between Schwann cells and regenerating axons. Functional assessment following transplantation of these grafts awaits further study.

Microglial Involvement in the Neural Regeneration

At present, microglial-neuronal interactions can be construed as a two-edged sword, due in part to two very different experimental approaches that have been employed *in vivo* and *in vitro* (see Streit, 1993). Therefore, to address the disparate results of these fundamentally different paradigms for characterizing microglial function, the primary focus of this thesis is on the examination of the cellular and neuritic responses to cultured microglia transplanted into the injured, adult rat spinal cord. Unlike the transplantation paradigms outlined above, cultured microglia have not yet been transplanted into the CNS to study their cellular interactions, migratory capabilities, differentiation or effects on the injured CNS. This is in spite of the demonstration that macrophages injected into peripheral wounds increase the rate of healing and reduce scarring (Danon et al., 1989).

Synopsis of the Evolution of this Dissertation

For general perspective, this thesis evolved from preliminary experiments that were designed to investigate the involvement of microglia

in the process of neural allograft rejection in the CNS. Recent work in cellular neuroimmunology has culminated in a new concept that views microglia as a network of intrinsic, immunocompetent cells of the CNS (Graeber and Streit, 1990). This sheds new light on the longstanding belief that the CNS is an immunologically privileged system, and may explain reasons for rejection. Immunohistochemical studies examining the expression of major histocompatibility complex (MHC) molecules in both normal and injured CNS tissue have shown that the principle cell type expressing MHC antigens is the microglial cell (Matsumoto et al., 1986; McGeer et al., 1988; Kono et al., 1989; Streit et al., 1989; Gehrmann et al., 1992; Morioko et al., 1992; Sedgwick et al., 1993). There is also compelling evidence that the immunocompetent microglia are the antigen-presenting cells (APCs) of the CNS and are thus responsible for initiating antigen-directed immune reactions mediated by T-lymphocytes that result in graft rejection (Frei et al., 1987; Hickey and Kimura, 1988; Streit et al., 1988; Poltorak and Freed, 1989; Graeber and Streit, 1990; Lawrence et al., 1990).

Therefore, experimental strategies were developed to deplete cells capable of MHC expression (i.e., microglia) from allogeneic neural suspension grafts. During the development of the microglial depletion/isolation procedures, the question arose as to what effect such depletion would have on axonal elongation either from embryonic CNS grafts, the host, or both. Review of the literature underscored that nothing was actually known about the effects of microglia on neuritic outgrowth in the injured CNS.

Accordingly, the experimental studies to be described in subsequent chapters represent the first attempts at investigating the role of microglia/brain macrophages in spinal cord regeneration. A primary issue in this regard relates to the mode of delivery. In the first set of experiments

(Chapter 2) this entailed the use of biodegradable polymeric tubes containing a gelfoam matrix that could be seeded with microglia derived from tissue culture. While some interesting observations were obtained pertaining to a possible supportive role of microglia, there was pronounced inflammation occurring around the polymeric carrier tubes that clouded interpretation of the control results. Another series of experiments (Chapter 3) was thus conducted in which a more stable support tube was used with which inflammation was not associated. The results of both investigations collectively challenge the view, noted earlier, that microglia exert a totally adverse effect on regeneration. In addition, some findings serendipitously indicated that the microglial implants might modulate astroglial responses to spinal cord injury, as well as the distribution of at least one ECM molecule (i.e., laminin). Finally, the question of reliable prelabelling of implanted cells was investigated (Chapter 4) as this is one of the more challenging technical issues associated with the transplantation of cells that have endogenous counterparts in the host CNS. Thus, some theoretical insights pertaining to neuron-glial and glial-glial interactions have emerged that set the stage for future experimental analyses (Chapter 5).

CHAPTER 2
TRANSPLANTATION OF CULTURED MICROGLIA INTO THE INJURED RAT SPINAL CORD: EVIDENCE FOR NEURITE GROWTH-PROMOTING ACTIVITY

Introduction

Microglia are known to secrete a variety of cytokines and growth factors, such as interleukin-1- (IL-1-) and IL-6-like molecules (Giulian & Baker, 1985; Giulian et al., 1986; Remick et al., 1988; Hetier et al., 1988; Frei et al., 1989; Woodroofe et al., 1991; Ganter et al., 1992), tumor necrosis factor (TNFα) (Frei et al., 1987; Remick et al., 1988; Woodroofe et al., 1991), transforming growth factor-beta (TGF-β) (Constam et al., 1992; Lindholm et al., 1992; Finch et al., 1993; Kiefer et al., 1993), basic fibroblast growth factor (bFGF) (Shimojo et al., 1991), and nerve growth factor (NGF) (Mallat et al., 1989; Lindholm et al., 1992). Since all these substances play interactive roles in tissue repair and nerve regeneration, several investigations have begun to explore neurotrophic and/or neurotropic properties of microglia under a variety of experimental conditions. Some results have demonstrated that microglia or microglia-conditioned medium can promote the survival of CNS neurons, as well as enhance their elaboration of neuritic extensions (Nakajima et al., 1989; 1993; Nagata et al., 1993a,b; Zhang and Federoff, 1993; Chamak et al., 1994). Whether these effects can be attributed to the secretion of cytokines/growth factors or to the deposition of extracellular matrix (ECM) molecules by microglia remains to be determined.

However, the presence and abundance of microglia/brain macrophages at sites of insult within the CNS has led others to propose that these cells may exert more destructive effects (Banati et al., 1993). For example they could exacerbate lesions through the production of putative cytotoxic molecules such as superoxide anions, glutamate and nitric oxide (Colton & Gilbert, 1987; Piani et al., 1991; Chao et al., 1992). It also has been reported that activation of microglia can lead to the *in vitro* production of low molecular weight, neurotoxic substances (Giulian, 1990; Giulian et al., 1993a,b).

In light of the conflicting views about microglial involvement in both CNS pathology and regeneration, the present study was designed to test whether cultured microglia exhibit any neurite growth-promoting actions after being grafted into the injured rat spinal cord. Whether microglia can have a modulatory effect on other glial (e.g., astrocytic) responses to trauma was another issue of particular interest. In addition, how microglia may influence the distribution of the ECM molecule laminin, commonly found around blood vessels and along the external glial limiting membrane of the normal CNS (Sanes, 1983), was also investigated. The reasoning stems from the association of laminin with astrocytes after invasive mechanical lesions at the interface between the cicatrix and blood vessels, and at reconstituted subpial surfaces (Liesi et al., 1984; Bernstein et al., 1985). Our results suggest that both grafted, as well as endogenous microglia that are present in regions of inflammation can be compatible with growing neurites and that their modulation of the microenvironment and gliotic responses may account for these observations. Portions of this study have been previously summarized (Rabchevsky et al., 1994; 1995).

Methods and Materials

Isolation of Microglia

Neonatal Sprague-Dawley rat brains were stripped of surrounding meninges in Solution "D" (containing 0.137 M NaCl, 5.4 mM KCl, 0.02 mM NaH_2PO_4, 0.02 mM KH_2PO_4, 5.5 mM Dextrose, 58.5 mM Sucrose, $1x10^6$ u. Penicillin G-1.0g Streptomycin (Sigma) and 2.5mg Fungizone (Gibco) in 10 ml distilled H_2O, pH 7.6). The tissue was then minced with a scalpel and resuspended in 20 ml of Solution "D" to which 0.15 % trypsin (Worthington) was added,and gently agitated on an orbital shaker at 37°C for 20 min. DNase (Sigma; 400 kunitz) was then added and the suspension was shaken for another 10 min. An equal volume of DMEM (pH 7.2), supplemented with 10% fetal bovine serum (FBS), was used to quench enzymatic activity. The suspension was subsequently filtered through a 130 µm Nitex filter. The filtrate was pelleted (400 x g, 10 min.), resuspended with 10 ml complete media, then filtered through a 40 µm Nitex filter. The cell concentration was adjusted to ~$1x10^6$ cells/ml of complete medium. Then 10 ml of suspension was added to individual poly-l-lysine-coated 75 cm^2 flasks before placing them into a humidified incubator set at 37°C, 8% CO_2 - 92% atmosphere for 3 days. The conditioned medium was replaced with complete medium, and 1 week later microglia were isolated from conditioned medium every 3-4 days by simply pelleting floating cells collected in the supernatants.

Characterization of "Floating Cells"

After plating, approximately 90% of floating cells were microglia as evidenced by labeling with *Griffonia simplicifolia* I-B_4 isolectin (GSI-B_4; Sigma) (Streit, 1990) or DiI-labeled acetoacetylated LDL (DiI-ac-LDL; Biomedical Technologies, Inc.) (Giulian et al., 1986). Removing non-adherent

cells after 1 hr. @ 37^{0}C yields >95% enrichment. Viability of the cells before transplantation was assessed with Trypan Blue to be >95%.

Experimental groups

Two strategies were employed to introduce cultured microglia into the spinal cords, each of which utilized a gelfoam (Upjohn Co.; Kalamazoo, MI) matrix that could be impregnated with enriched microglia. One method was the implantation of gelfoam alone (GF), and the other presented the matrix within biodegradable polymeric tubes (GF+PT) to provide a potential conduit through which neurites could grow. Control implants which consisted of cell-free (i.e., medium-only) gelfoam (GF-DMEM) paralleled these two experimental groups. The microglia-impregnated gelfoam grafts (GF-M-DBM; gelfoam and microglia-derived brain macrophages) were implanted into injured, adult rat spinal cords for 2 (N=3) and 3 (N=4) weeks with two control implants at each interval. The gelfoam-filled polymeric tubes saturated with microglia (GF+PT-M-DBM) were also examined at 2 (N=2) and 3 (N=3) weeks after transplantation. These were paralleled by control tube implants (GF+PT-DMEM) of 2 (N=3) and 3 (N=2) weeks.

Microglial Impregnation of Gelfoam/Polymeric Implants

Polymeric tubes (Medisorb Technologies International, L.P.; 5050 DL), consisting of 1:1 polylactic:polyglycolic acid (~3 mm x 1 mm), were UV sterilized and filled with sterile gelfoam. Placing the tubes upright within a sterile petri dish, a suspension of microglia (~50,000 cells/µl) was slowly injected with a sterile 22 gauge Hamilton syringe until the gelfoam core was saturated. Tubes were kept in this position for several minutes before inverting them and repeating this procedure. The saturated tubes were then placed on their sides and immersed in complete medium for subsequent incubation at 37^{0}C (>4 hrs.) before transplantation. Gelfoam-only implants

(~1mm^3) were seeded with microglia similarly and incubated in complete medium until transplantation. Control, cell-free gelfoam was soaked only in complete medium in both types of implants.

Surgical Procedures

Adult female (150-300g) Sprague-Dawley rats (N=21) were anesthetized with Ketamine (90 mg/kg, i.p.) and Xylazine (10 mg/kg, i.p.) before a laminectomy was performed at vertebral T_{11}. A midline dural incision was made for approximately 2-4 mm length. Suction was then used to create a dorsolateral cavity ~3 mm long. After hemostasis was achieved, the experimental (N=12) or control (N=9) implants were put into the damaged spinal cord after which the dural incision was sutured with 10-0 Ethicon. Gelfoam was laid on top of the dura before the muscle and skin openings were closed. Bladders were expressed as required.

Tissue Processing

After 2-3 weeks, the rats were overdosed with 4% chloral hydrate before transcardial perfusion with phosphate-buffered saline (PBS) containing heparin and sodium nitrate, followed by 4% paraformaldehyde in PBS (PF). The fixed spinal cords were immediately dissected and post-fixed in 4% PF for 30 min. before transferring them to 30% sucrose/PBS for several days at 4^{0}C. Specimens were then embedded in OCT mounting media, frozen in liquid nitrogen-cooled isopentane, and stored at -70^{0}C until cryosectioning (20-30μm) and mounting onto gelatin-coated slides.

Microglial cultures were maintained either on glass coverslips or in 35 mm plastic petri dishes. Before staining, cultures were washed twice with PBS and then fixed with 4% PF for 20 min., or acetone for 10 minutes.

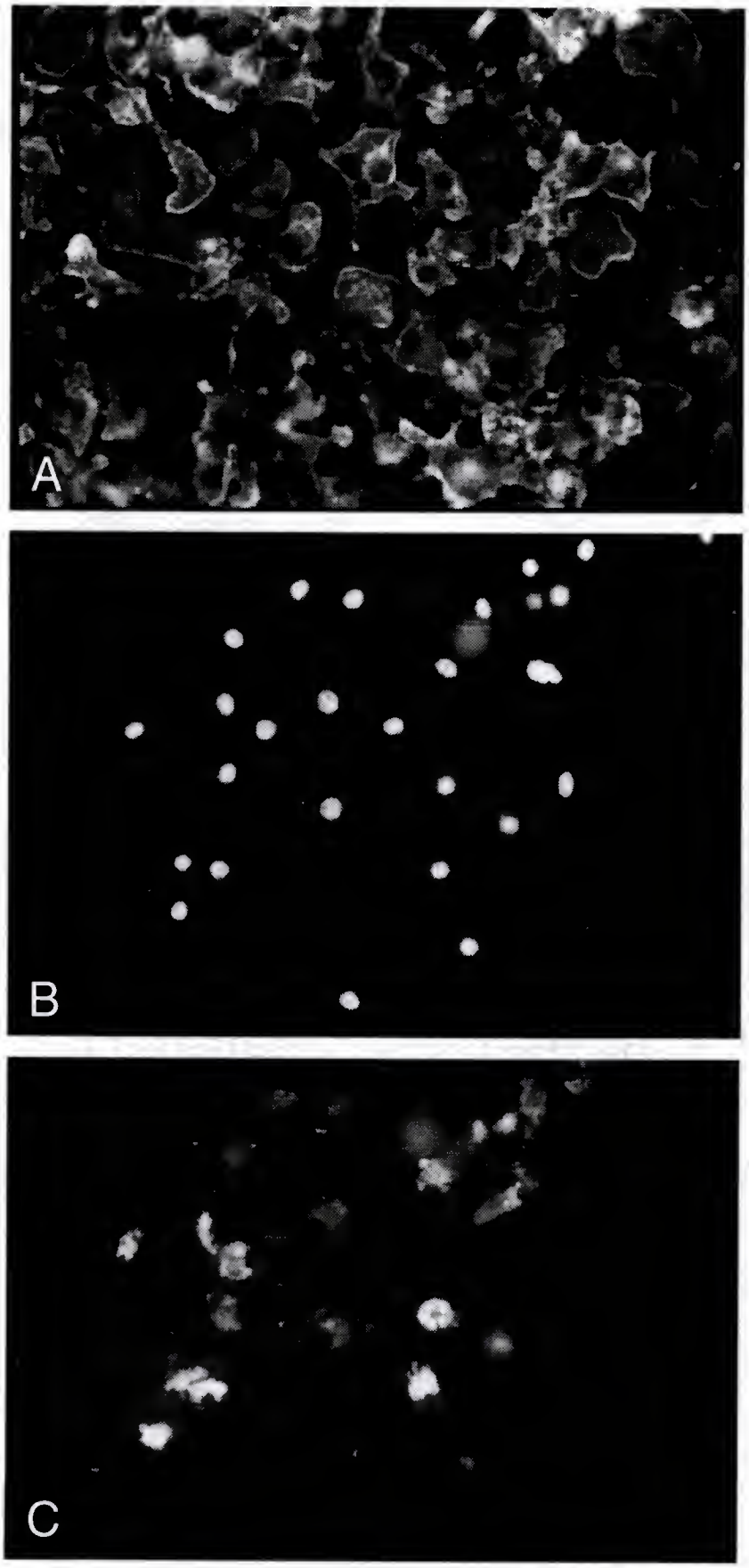
Immunofluorescent Cytochemistry

In Situ. Before applying primary antibodies to air-dried sections on slides, the sections were incubated for 30 min. with PBS containing 3% serum from the species in which the secondary antibodies were raised. Then, primary antibodies diluted in PBS containing 1% of the same serum were applied overnight at 4^0C [OX-42 (Serotec; 1:200); mouse anti-neurofilament (NaP4-phosphorylated; 1:400); mouse anti-GFAP (Sigma; 1:400); rabbit anti-laminin, (Sigma; 1:500)]. After several washes with PBS, secondary antibodies in PBS were applied for 2 hours at room temperature [biotinylated anti-mouse/rabbit (Vector; 1:300; 1:500); goat anti-mouse IgG-TRITC (Jackson; 1:400)]. If there was a tertiary antibody [streptavidin-FITC/TRITC (Vector; 1:300)], the procedure was repeated, incubating with this antibody in PBS for at least 1 hour at room temperature. Control slides were incubated with PBS/1% serum without primary antibodies.

When all incubations were complete, the slides were washed several times with PBS and then coverslipped with glycerin/PBS (Citifluor; Canterbury, U.K.) mounting medium for viewing under a Zeiss microscope equipped with epifluorescent illumination (Axiophot filters: #01-UV; #09-FITC; #14-RITC). Hoechst 33342 (Sigma) nuclear dye was added to the mounting media (5μM) in order to get a better estimate of cellular profiles. Coverslips were sealed onto the slides with Cutex nail hardener (#01).

In Vitro. After fixation, the dishes/coverslips were washed with PBS before incubating the cultures with primary antibodies or GSI-B4-FITC lectin (Sigma; 10 μg/ml) in PBS for 2 hr. at room temperature. The dishes/coverslips were then washed several times with PBS and incubated with secondary antibodies in PBS for 2 hrs. The remaining procedures were done as described above.

Fig. 2-1. Immunofluorescent staining of microglia/brain macrophages *in vitro*. A. Acetone-fixed microglia cultured on a glass coverslip (6 DIV) were stained with OX-42, an antibody recognizing the CR3 complement receptor expressed on macrophages (X320). B-C represent images of 4% PF-fixed microglia impregnated in gelfoam (same field viewed through UV and FITC filters, respectively) (X320). B. Hoechst 33342 dye in the mounting media demonstrates the viability of the cells within the gelfoam matrix after 7 days *in vitro*. C. GSI-B4-FITC lectin stain identifies these cells as microglia.


A
B
C

Results

Characterization of Microglia-Impregnated Gelfoam *In Vitro*

Isolated microglia were cultured and histochemically identified with OX-42 antibody against their CR3 receptor (Graeber et al., 1988; Fagan and Gage, 1990) (Fig. 2-1A) and with GSI-B4 isolectin (Streit et al., 1988; Streit, 1990) (Fig. 2-1C). They were cultured on pieces of gelfoam to establish that the cells were adhering and surviving in the matrix (Figs. 2-1B,C). Additionally, microglia grew well *in vitro* on the polymeric material comprising the tube walls and remained attached to it for up to 28 days *in vitro* (DIV) while the polymer was undergoing degradation. In retrospect, however, one limitation of the gelfoam was that uniform impregnation could not be routinely achieved since cultured cells were not always found in the central regions of the gelatinous matrices.

For the purposes of this report, the term "microglia-derived brain macrophage" (M-DBM) is used as a more accurate description of the nature of cultured microglial cells because they are typically isolated from primary mixed brain cultures as brain macrophages. These cells form in response to the tissue debris generated during the culture preparation. Thus, using the *in vivo* terminology, purified microglia *in vitro* are microglia which have already undergone activation to the macrophage state (Streit, 1993).

Neuritic Ingrowth and OX-42-IR Distribution in GF-DMEM and GF+PT Control Implants

Immunostaining for the phosphorylated, heavy subunit of neurofilament (NaP4) was used to demonstrate the extension of neuritic processes into M-DBM-seeded or control GF matrices. Immunoreactivity (NF-IR) was clearly seen in axons of surrounding host white and gray matter,

Fig. 2-2. Control, cell-free gelfoam as observed 3 weeks after implantation. A. Horizontal section through a spinal cord implanted with gelfoam alone shows scant inflammation and sparse OX-42-IR in the matrix (X40). B. Anti-neurofilament staining of the same implant (inset in A) at the gelfoam-host border (arrowheads) shows scant neurofilament staining (arrows), despite host cellular infiltration (X120). C. Adjacent section to A, showing that laminin-IR is primarily restricted to the host cord surrounding the implant and to the periphery of the gelfoam.

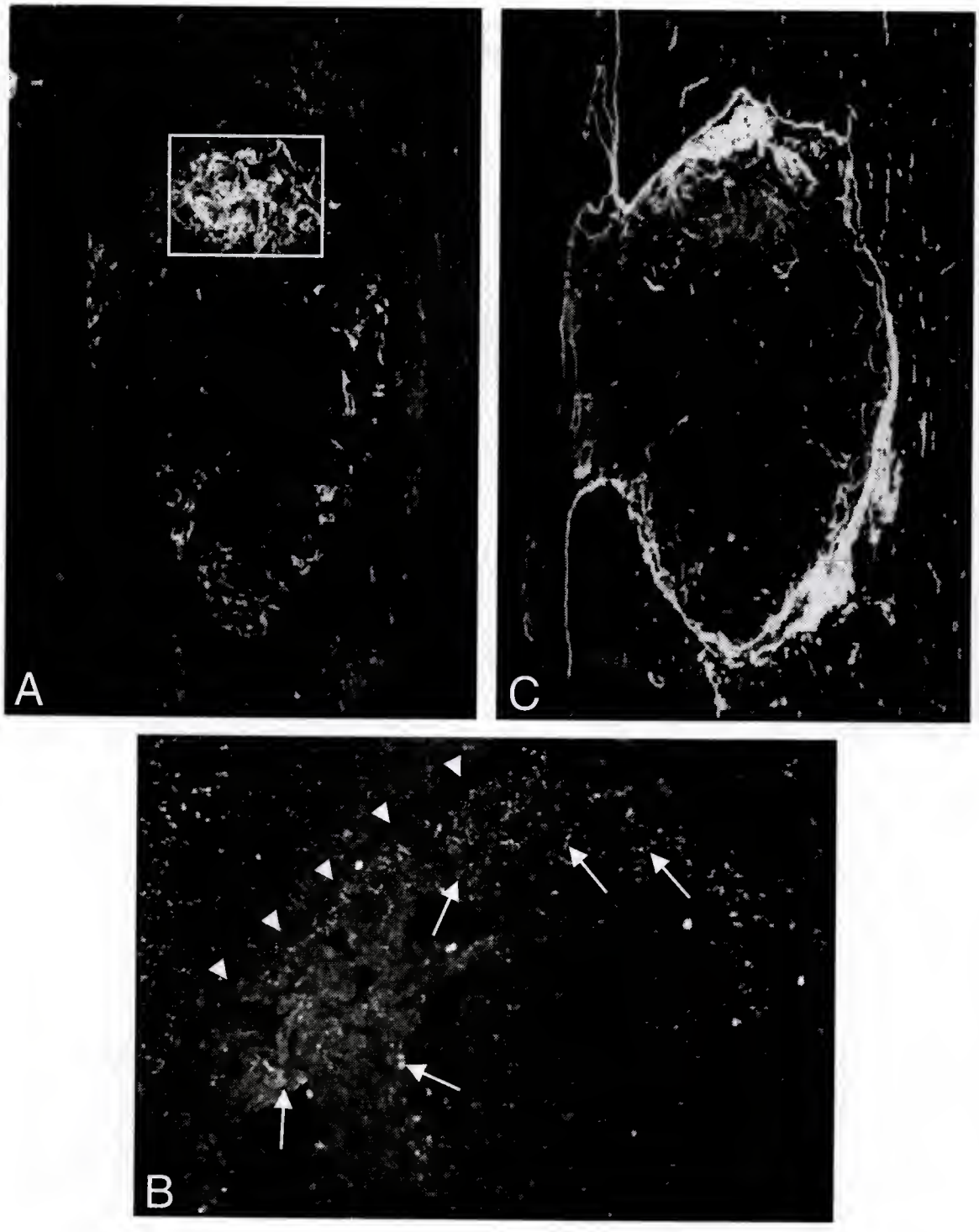

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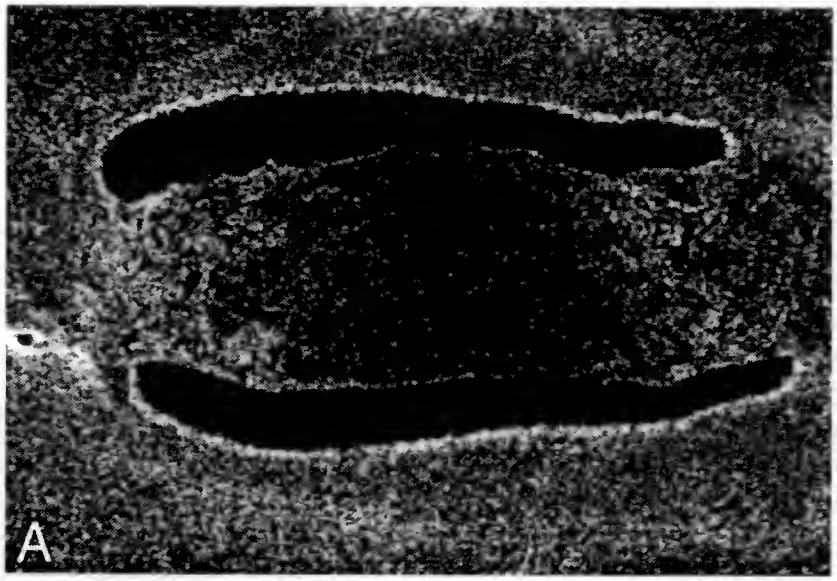
Fig. 2-3. Control biodegradable polymeric implants filled with gelfoam soaked in culture medium 2-3 weeks after implantation. A. Hoechst 33342 staining of a horizontal section through the lumen of a gelfoam-only tube demonstrates the accumulation of cells around the implant, especially along the walls and within the rostral and caudal parts of the matrix after 2 weeks (X40). B. An adjacent section to A immunostained with OX-42 antibody. Numerous immunoreactive elements are seen along the tube walls and a prominent infiltration of OX-42-IR cells is indicated in the matrix. C. A section at the rostral tube implant-spinal cord interface (arrowheads outline implant walls) stained with neurofilament antiserum after 3 weeks (X80). Some neuritic processes (arrows) are seen extending into the gelfoam matrix which was shown by Hoechst 33342 dye and OX-42 staining to be heavily infiltrated with host cells. Note that while the wall thickness appears much less than in A and B, this is due to a cryosectioning artifact in which the integrity of the walls in many of the implants cracked and separated (A,B) after thawing onto gelatin-coated slides.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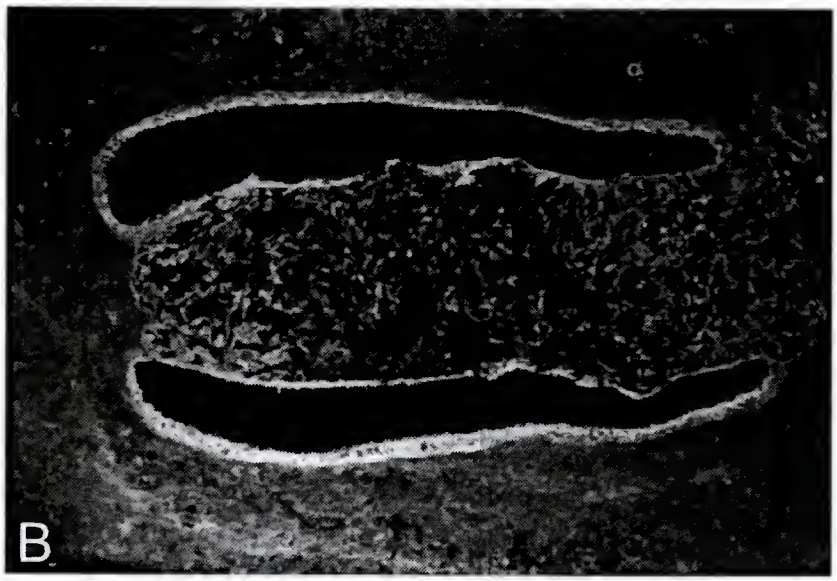
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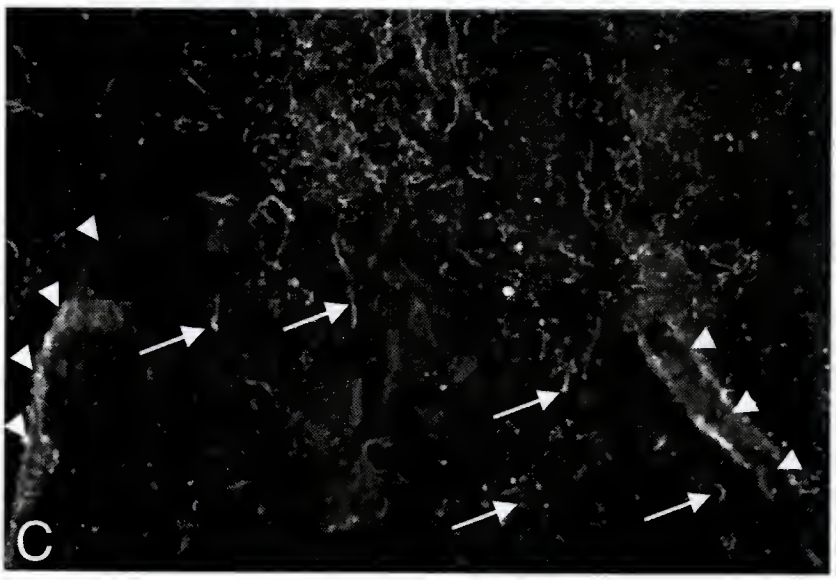
C

and in the soma of neurons surrounding the implants which were perhaps damaged during transplantation (Drager and Hofbauer, 1984).

None of the GF-DMEM implants had exhibited prominent neuritic ingrowth. Fibers were seen in the host tissue juxtaposed to the implants, but few neurites were seen in the peripheral margins of the matrices (Fig. 2-2C). After two and three weeks, OX-42-IR elements were primarily restricted to cellular infiltrates at the periphery of these implants, and a more pronounced occupation of the graft matrix, which included other unidentified cells (Hoechst dye), was apparent after three weeks (Fig. 2-2A).

Control GF+PT implants, however, presented a more complex result in that a modest degree of neuritic ingrowth was seen in some cases. When present, these NF-IR profiles were primarily restricted to the rostral and caudal ends of the polymeric tubes (Fig. 2-3C). By two weeks, the implants were heavily infiltrated by host-derived cellular elements, many of which exhibited OX-42-IR (Figs. 2-3A,B). These infiltrates completely filled the tubes after three weeks, and such cell-rich control implants showed the most robust ingrowth of neurofilament-IR processes that were usually seen in conjunction with OX-42-IR cells.

Neuritic Ingrowth and OX-42-IR Distribution in
GF and GF+PT Implants Seeded with M-DBM

Unlike the control gelfoam implants, the matrices impregnated with cultured microglia displayed a relatively homogeneous distribution of OX-42-IR cells throughout the matrix after two and three weeks, except at the center of the implants which tended to be relatively refractory to the cell seeding procedure (Fig. 2-4A). The OX-42-IR profiles within the matrix had a unique, swirling pattern reflecting M-DBM aggregates formed during the seeding

Fig. 2-4. Intraspinal implants of gelfoam impregnated with cultured microglia examined 2 weeks after grafting. A. Horizontal section through an implant demonstrating OX-42-IR elements dispersed throughout the matrix (X40). B. An adjacent section to A demonstrating the lack of GFAP-IR cells in an implant; however, the gelfoam insert was completely lined by a wall of GFAP-IR processes (arrows). C and D. Microglial-impregnated gelfoam implants double-stained with anti-OX-42 (C) and anti-laminin (D) antibodies. While some of the laminin-IR appeared to be associated with microvascular elements in the surrounding host tissue, there was a very similar distribution between the immunoreactive profiles within the microglia-impreganted matrices (arrows) (X120).

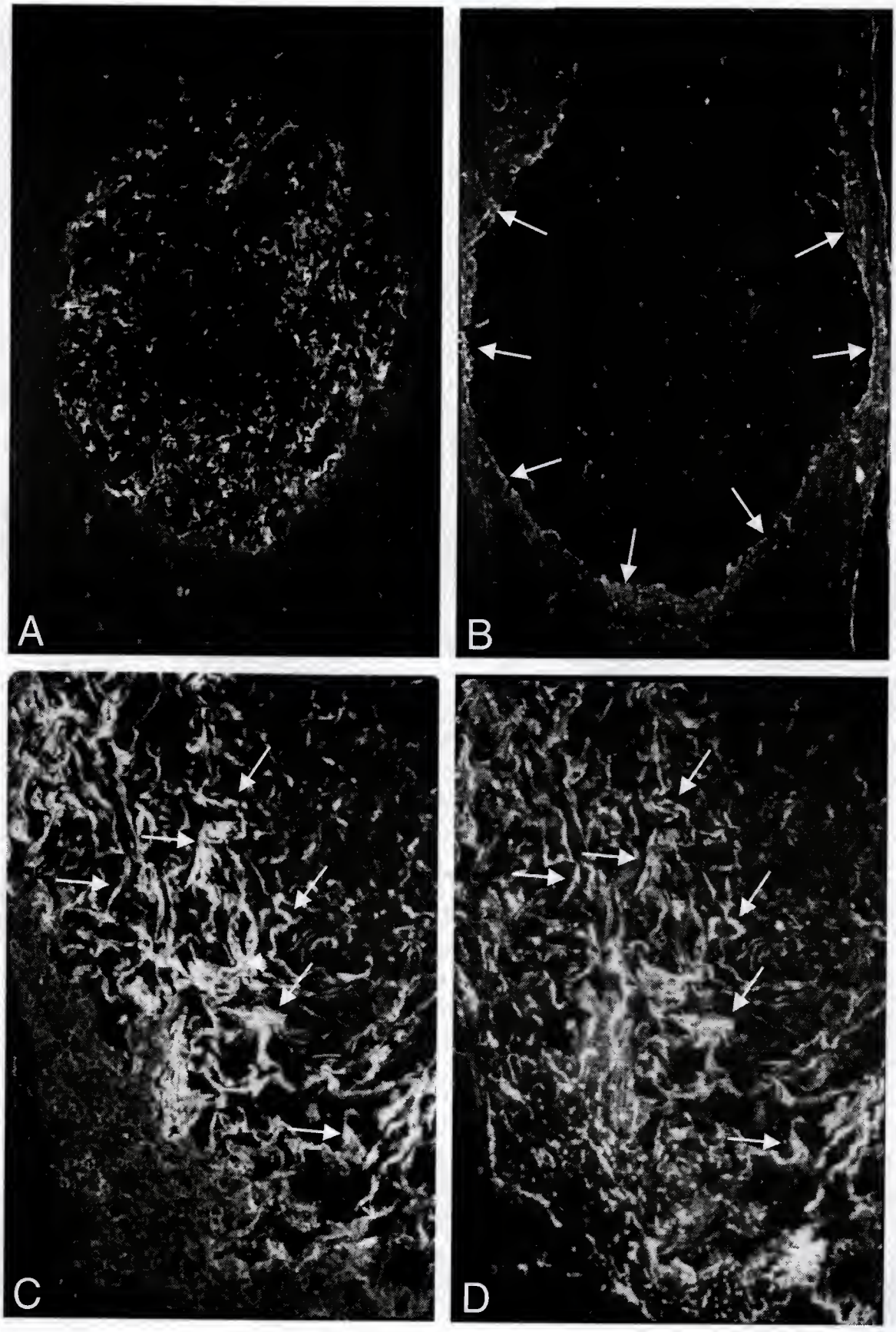
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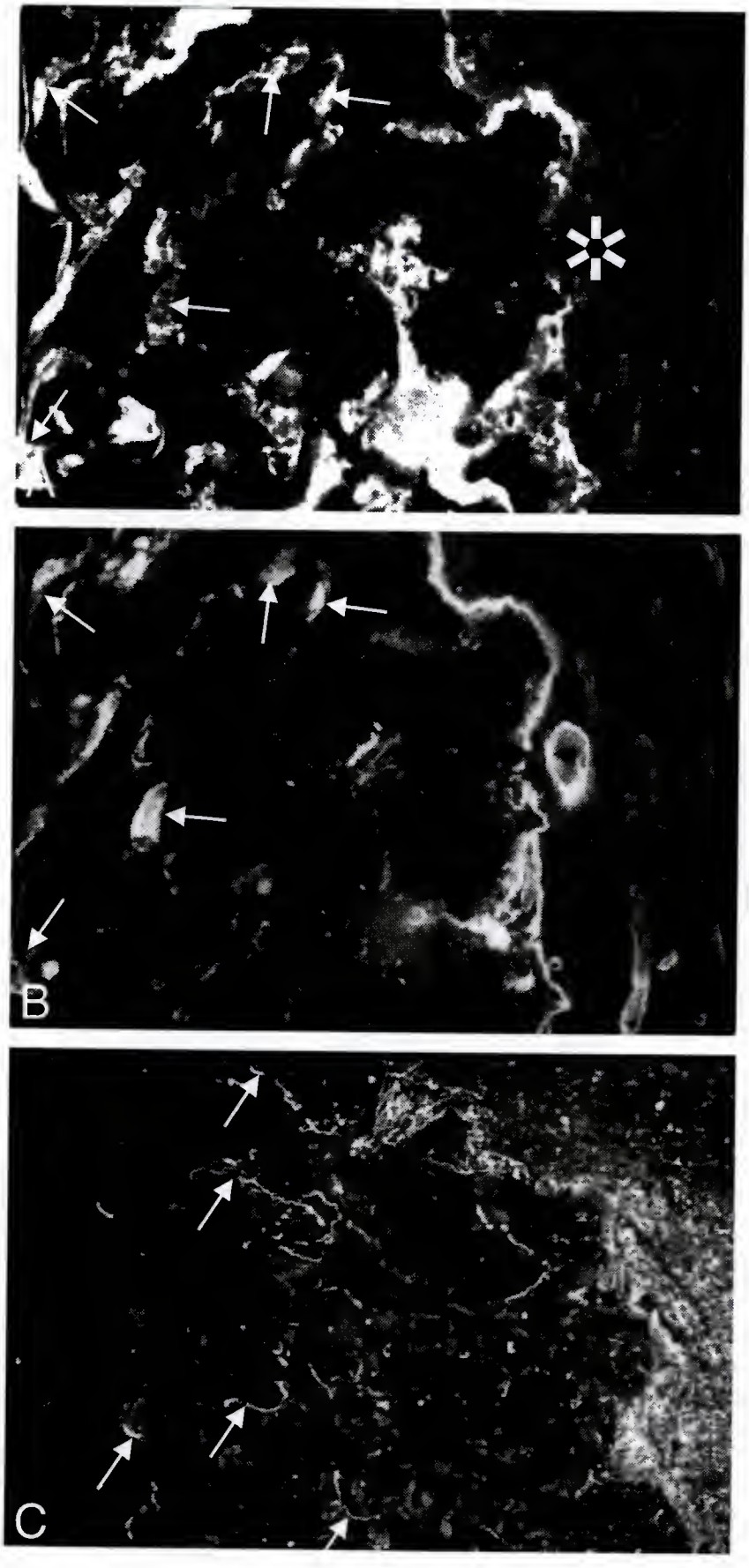
Fig. 2-5. Higher magnification of the graft-host border in Fig. 2-5 (C,D) demonstrating the sparse, but consistent colocalization (arrows) of OX-42-IR cells (A) with laminin-IR elements within the matrix (B). Note the lack of OX-42-IR in the host vasculature (asterisk), serving as an internal control. C. Adjacent section demonstrates pronounced neuritic ingrowth (arrows) into the microglia-impregnated gelfoam (X320).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procedure. Neuritic ingrowth was seen throughout these impregnated matrices in register with many of these aggregates (Fig. 2-5C).

After two and three weeks, GF+PT implants seeded with M-DBM also showed homogeneous distributions of OX-42-IR cells throughout their matrices (Fig. 2-7A), and numerous NF-IR profiles were seen crossing at the rostral and caudal host-tube interfaces (Figs. 2-6B, 2-7B). This neuritic ingrowth was consistently more robust than that seen in any of the control GF+PT implants, even after three weeks. However, while the NF-IR processes were found in all orientations throughout the matrices (Fig. 2-7B), some were seen coursing along the inner walls of the tube (Fig. 2-6B), similar to those seen in control GF+PT implants with heavy infiltrates.

Surrounding Host Inflammatory Responses and NF-IR Distributions

Nissl stains and Hoechst 33342 dye revealed that implants of gelfoam, with or without seeded M-DBM, induced relatively little inflammation. In striking contrast, all the GF+PT implants demonstrated a robust host inflammatory response characterized by a multilayered disposition of cells surrounding the exterior of each tube that could be revealed by OX-42 immunostaining (Figs. 2-3A,B). Many of these cells also appeared to stream into the gelfoam matrix at the open ends of the tubes, and they lined the inner walls as well. Interestingly, NF-IR profiles were often seen coursing through regions of inflammation in the surrounding tissue that were partially characterized by OX-42-IR cells (Figs. 2-8A-C).

Astroglial and Laminin Distributions

A narrow, but well-defined, zone of astroglial cells and processes, denoted by GFAP-IR, was seen encapsulating the control GF-DMEM implants; however, no GFAP-IR elements were extended into the matrix. Anti-laminin immunohistochemistry revealed characteristic staining of blood

Fig. 2-6. Horizontal sections of polymeric tube implants which contained microglial-impregnated gelfoam as seen 2 weeks after being implanted into the injured rat spinal cord (arrowheads indicate inner tube walls). A. Photograph of GF+PT implant impregnated with prelabeled microglia (Hoechst 33342) that are seen scattered throughout the matrix (white dots). Adjacent sections stained with GSI-B4 lectin confirmed that the nuclear profiles were virtually all microglia (not shown). B. The same field shown in A that was immunostained with anti-neurofilament (NF). Arrows indicate NF-IR profiles that were seen extending into the gelfoam interior at the implant-host interface (X60). C. Higher magnification of graft-host border in an adjacent section demonstrates few GFAP-IR processes penetrating the gelfoam (X80), and no GFAP-IR elements were found in the matrices or juxtaposed to the exterior wall surfaces.

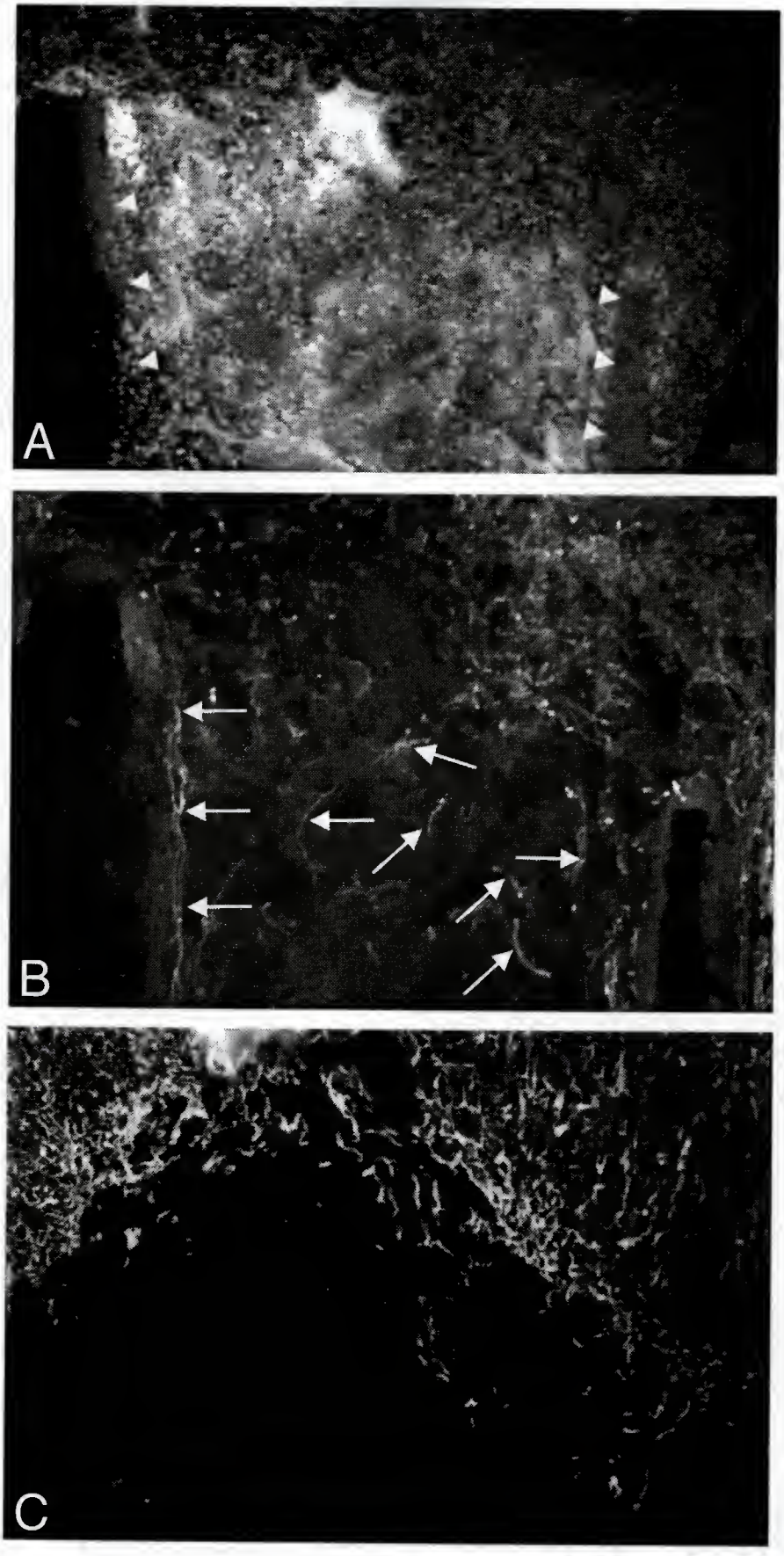
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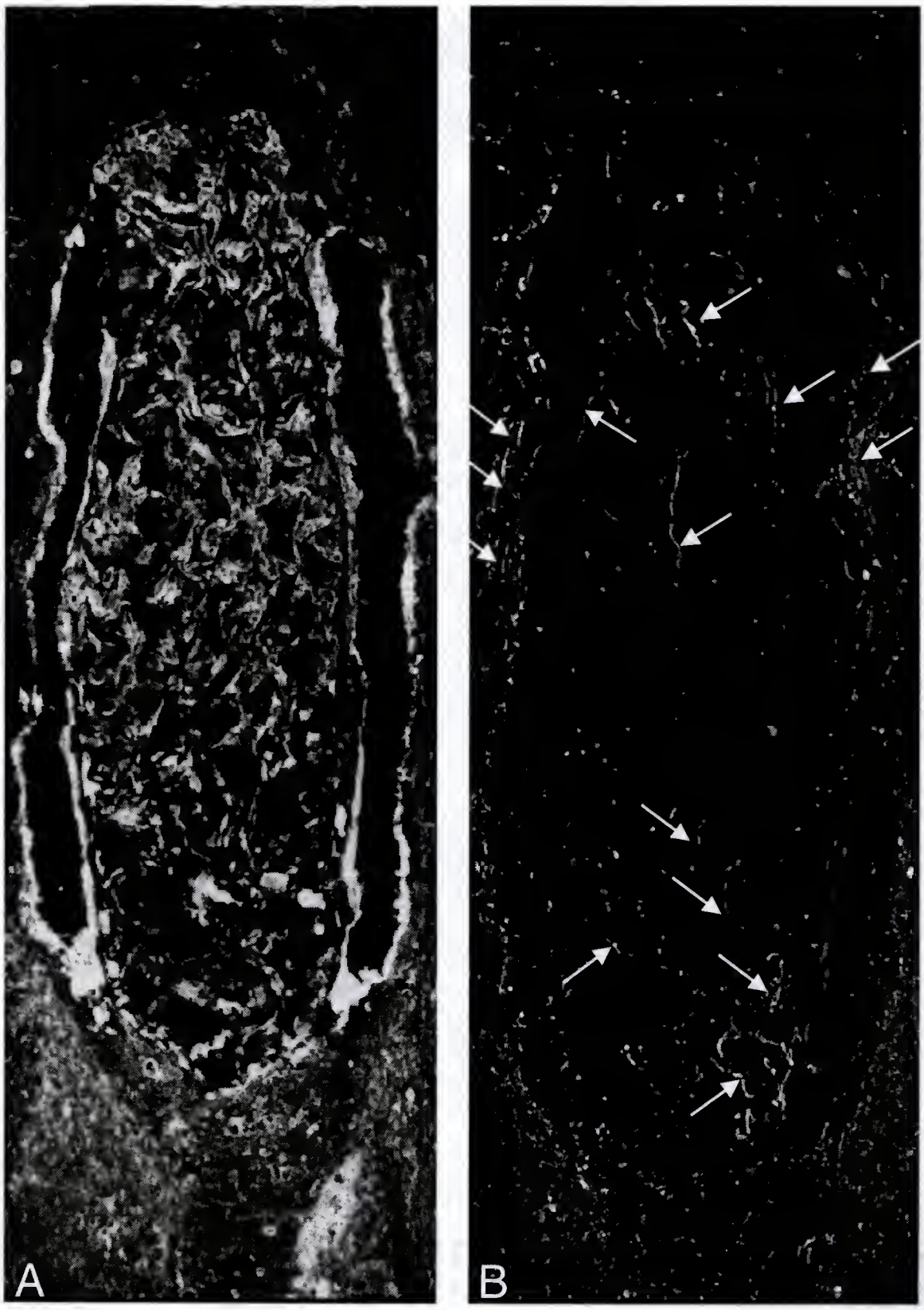
Fig. 2-7. Shown are adjacent horizontal sections through a polymeric tube implant containing gelfoam impregnated with cultured microglia (3 weeks after implantation). A. Intense OX-42-IR is seen throughout the microglia-impregnated gelfoam matrix (X50). B. Anti-neurofilament staining showed pronounced neuritic ingrowth from both rostral and caudal segments of the spinal cord and numerous neuritic processes coursing along the outer walls of the degrading tube (arrows).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vessels and subpial surfaces in the spinal cord, and intense laminin-IR also was seen immediately surrounding the gelfoam (Fig. 2-2C). Such immunoreactivity, however, was scarcely detected in the interior of these implants. Similarly, GFAP-IR was exclusively restricted to the host spinal cord surrounding the control GF+PT implants (Fig. 2-6C). These astroglia were separated from the exterior surfaces by OX-42-IR cells intrinsic to the inflammatory responses around these tubes as noted above.

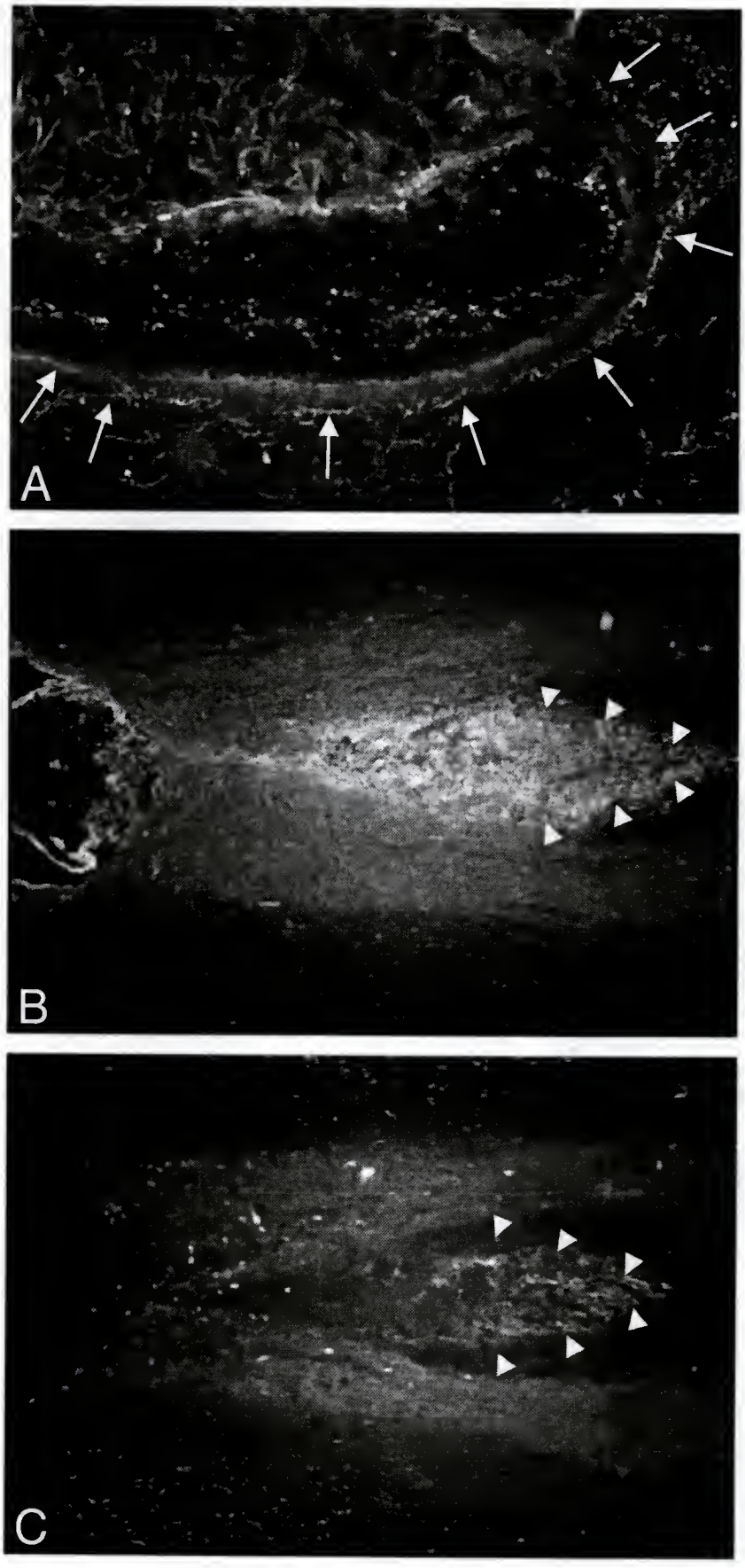
Similar to control GF-DMEM, the M-DBM-seeded GF implants were also surrounded by a wall of astroglial cells and their processes, and no GFAP-IR elements were seen within these matrices (Fig. 2-4B). In contrast, however, to control implants intense laminin-IR was seen throughout these grafts in register with many of the OX-42-IR cellular profiles (Figs. 2-4C,D). While some of the laminin-IR appeared to be associated with microvascular elements, higher magnification revealed some colocalization of OX-42-IR cells with laminin-IR elements (Figs. 2-5A,B).

Discussion

Permissive vs. Non-Permissive Effects of Microglia on Axonal Elongation

This study represents an initial investigation of the influences of microglia-derived brain macrophages (herein referred to as microglia for simplicity) on axonal elongation in the injured spinal cord using a transplantation approach in which cells isolated *in vitro* were introduced into lesioned cavities *in situ*. The hypothetical framework of this study is based on the contrasting effects that microglia have been proposed to have in the injured CNS. In particular, the interest was in determining whether microglia would adversely affect axonal elongation based on the speculation

Fig. 2-8. Characterization of inflammatory regions in longitudinal, horizontal sections through spinal cords implanted with control tubes after 2 weeks. A. Neurofilament staining was seen along the outer walls of the degrading polymeric tubes (arrows) that were marked by intense OX-42-IR (see Fig. 2-3b), and virtually no neurites were seen entering the Gelfoam (X120). B. An area of inflammation (OX-42-IR) in the dorsal columns rostral to the tube (arrowheads) demonstrates the presence of neurofilament staining (C) within this region in an adjacent section (X20).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of their putative neurotoxic effects (Colton & Gilbert, 1987; Piani et al., 1991; Chao et al., 1992; Giulian et al., 1993a,b; Banati et al., 1993). In principle, any substantial degree of neuritic elongation in the presence of these cells would constitute a challenge to this hypothesis.

In that regard, it was found first that a qualitatively greater ingrowth of NF-IR profiles was seen in gelfoam matrices that had been seeded with microglia prior to grafting. This occurred irrespective of whether or not this material was partially encased in biodegradable polymeric tubes. Some neuritic elongation also was observed, unexpectedly, into control gelfoam (i.e., unseeded) when placed into the spinal cord in conjunction with the polymer casing. The tubular prosthetic was used to provide a more structured environment for analyzing axonal growth patterns analogous to what has been done in studies of other grafted cell types into the injured CNS (Kromer and Cornbrooks, 1985; Hoffman et al., 1993; Xu et al., 1995). However, the extent of NF-IR elements entering some control GF+PT implants was modest compared to the cell-seeded counterparts, and the distribution of profiles was usually restricted to the open ends of the tubes infiltrated by host elements. Interestingly, many NF-IR fibers appeared to coincide with the presence of OX-42-IR cells. It is tempting to speculate, therefore, that this result indicates endogenous, activated microglia also might be permissive to axonal elongation. Thus, the results obtained with cell-seeded matrices are not necessarily attributable to a unique property of microglia *in vitro*.

This is further borne out by the observation of NF-IR profiles in regions of dense OX-42-IR in the spinal cord immediately surrounding the polymeric tubes. The accumulation of these cells most likely reflects an inflammatory response elicited by the degradation of the tubes; no similar

inflammation was observed with any of the GF implants. It has been shown that changes in axonal growth-promoting properties of the CNS near lesion sites may be produced by brain macrophages that modify nonpermissive substrata (David et al., 1990). Thus, the indication of axonal elongation around the degrading tubes would be consistent with this view; however, further studies are necessary to demonstrate the specific temporal relationships between neuritic elongation and the establishment of the OX-42-IR cell microenvironment within the degrading polymeric tubes (Rabchevsky and Reier, in progress).

Distribution of GFAP- and Laminin-Immunoreactive Elements

One of the hallmark features of CNS injury is the re-establishment of an astroglial limiting membrane along regions of white and gray matter that become exposed to non-CNS tissue microenvironments. The magnitude of this glial response can vary relative to a variety of lesion conditions (Feringa et al., 1980; 1984; Liesi et al., 1984; Bernstein et al., 1985). Nevertheless, even a single layer of astrocytes has been thought to be sufficient enough to impede axonal elongation (Smith et al., 1986; McKeon et al., 1991). In the present study, a well-defined region of glial reactivity was indicated by GFAP immunostaining that essentially outlined the border of these implants. In the case of GF grafts, the glial zone was in proximity to the border of the matrix, whereas in the case of the GF+PT implants, regions of gliosis were separated from the implants by OX-42-IR cells.

Given the presence of astroglial reactivity in these transplant paradigms, it is of interest that evidence for neuritic ingrowth was observed. This leads to the possibility that microglia may modify either the geometry of a glial scar or certain properties of astroglia that are incompatible with axonal elongation (Smith et al., 1986; McKeon et al., 1991). Again, speculation needs

to be tempered since it cannot be determined from these studies whether fiber extension into the gelfoam matrices preceded development of the astroglial wall. Also, ultrastructural studies would serve to define the degree of integrity that such glial regions actually exhibit. Finally, it would be of interest to determine whether ingrowth continues at later time-points than examined in the present investigation.

There are many conceivable ways whereby astroglial responses could be modified by activated microglia. For example, it has been suggested that these cells may regulate astroglial proliferation and scar formation through the production of IL-1 and TGF-β1 (Giulian et al., 1986; Lindholm et al., 1992; Sievers et al., 1993). Another way could be related to the deposition of basal lamina (BL) which, in part, could be examined by way of laminin-IR. After CNS injury, a BL is formed along exposed regions of the brain or spinal cord which essentially mirrors the distribution of GFAP-IR processes (Matthews and Gelderd, 1979; Feringa et al., 1980; Liesi et al., 1984; Kromer and Cornbrooks, 1985). This BL, associated with the subpial endfeet of astrocytes, may interfere with axonal growth through a lesion either by being a physical barrier or conversely a favorable substratum to which axons prefer to remain adherent (Reier et al., 1983; 1988). It is possible that the astrocytes, developing into scar tissue, do not represent a physical barrier but a poor substratum because of a deficiency in surface molecules, ECM molecules, or soluble substances that must be provided to their external milieu for regeneration to occur (Schwartz et al., 1989). It thus becomes of interest that all control GF and GF+PT implants did not show laminin-IR in the matrix environment, whereas there was a pronounced distribution of laminin in matrices that were originally seeded with microglia *in vitro*. Thus, part of the neuritic extension into the gelfoam matrices may be due to the deposition of this ECM

molecule known to have pronounced neurite growth-promoting properties *in vitro* (Rogers et al., 1983; Lander et al., 1985).

Potential Growth-Promoting Influences of Other Cell/Tissue Types

The presence of laminin-IR in cell-seeded or host microglia infiltrated gelfoam implants raises the possibility that the ingrowth of NF-IR elements may not be directly related to axon-microglial interactions but, instead, to a microglial-induced infiltration of other cell types that have growth-promoting properties. While there were some examples of colocalization of OX-42- and laminin-IR, this was not of a magnitude sufficient enough to account for the degree of laminin-IR observed in the grafts. Furthermore, it has been reported that laminin is not produced by microglia grown *in vitro* (Chamak et al., 1994). Likewise, it is unlikely that laminin deposition reflected some redistribution of mature astroglia since no GFAP-IR elements were observed in any of the grafted matrices. Because this colocalization was unexpected, especially in the absence of any GFAP-IR cells which are known to express laminin, further controls may be necessary to repeat these results and to possibly identify the isoforms expressed (Liesi and Risteli, 1989).

By exclusion, endothelial and Schwann cells represent the most likely laminin-producing cell types that could be recruited from the host into the gelfoam matrices. Whether microglia exert an angiogenic effect as do their peripheral counterparts (Mustoe et al., 1987) is presently unknown. However, because no distinction can be made in culture between parenchymal and perivascular microglia, many of the OX-42-IR elements in the grafts may have been of a perivascular nature. However, while laminin immunostaining revealed distinct vascular elements in the host spinal cord, the pattern of laminin-IR in the gelfoam matrices was not as obvious. The ingrowth of vascular elements, however, remains a tangible possibility, and this cellular

terrain could also serve as one route of Schwann cell entry. This emphasis on laminin, however, does not exclude the possible involvement of other non-laminin-producing elements with neurite growth-promoting properties (e.g., immature mesenchymal cells) or the deposition of other ECM molecules.

Closing Comment

The present transplantation study has provided some examples of neuritic elongation in the presence of microglia/brain macrophages of both donor and host origin. While the specific role of these cells in promoting axonal elongation cannot be ascertained from the circumstantial observations, these initial findings argue against the proposed negative effects of microglia in CNS injury. In addition, these observations suggest that microglia may be instrumental in mobilizing cells that have more well-defined neurite growth-promoting properties. In that case, the continued expression of growth-promoting properties is a further indication that microglia do not cancel or suppress these effects.

CHAPTER 3
CELLULAR AND NEURITIC RESPONSES TO GRAFTS CONTAINING MICROGLIA OR ASTROCYTES IMPLANTED INTO THE LESIONED SPINAL CORDS OF ADULT RATS

Introduction

Axonal regeneration in the adult mammalian central nervous system (CNS) has long been viewed as being an abortive process (Ramon y Cajal, 1928). Over the years, this failure of regrowth has been attributed to a variety of mechanisms (McKeon et al., 1991; Reier et al., 1983; 1988; Schwab, 1990). Two of the more prominent, and not necessarily mutually exclusive, views is that the CNS either lacks cell types that can exert either neurotrophic or neurotropic influences or that the cellular composition of the CNS is non-permissive to axonal elongation. The fact that neurons intrinsic to various regions of the brain, spinal cord, or retina have the inherent ability to regrow damaged processes is now well recognized as the result of peripheral nervous system (PNS)-to-CNS grafting experiments (Aguayo et al., 1982; David and Aguayo, 1981; Richardson et al., 1980). This effect of PNS tissue has been ascribed primarily to the Schwann cell. More recent emphasis has been on the use of purified Schwann cell populations to promote axonal regeneration in the injured spinal cord (Paino and Bunge, 1991; Paino et al., 1994).

Thus, neuritic elongation can be evoked when the cellular microenvironment of the CNS is replaced by Schwann cells and other cellular components of the PNS. One CNS component that has been traditionally viewed as being incompatible with regeneration is the astrocyte

(Reier et al., 1983; 1988). Though the adverse effects of this cell on axonal outgrowth is still not universally accepted, there are several lines of evidence showing poor fiber extension in an astrogliotic environment both *in situ* and *in vitro* (Reier et al., 1983; 1988; Liuzzi and Lasek, 1987; Fawcett et al., 1989). Oligodendrocytes and the white matter which these cells produce represent another collective constituent that can render the CNS nonpermissive to regeneration (Schnell and Schwab, 1990; Schwab, 1990).

Another major cellular component of the CNS is the microglial cell; however, its role in relation to neuritic outgrowth has not been extensively studied. In this respect, differing views have emerged from *in vivo* and tissue culture studies to the extent that one perspective is that these cells may actually be compatible with fiber outgrowth (Nakajima et al., 1989; 1993; Nagata et al., 1993a,b; Streit, 1993; Zhang & Federoff, 1993; Chamak et al., 1994), whereas the other notion is that these cells may produce substances that can be toxic to neurons (Colton & Gilbert, 1987; Giulian, 1990; Piani et al., 1991; Chao et al., 1992; Banati et al., 1993). This issue was addressed in the preceding chapter by virtue of an intraspinal grafting approach in which microglia-derived brain macrophages were isolated *in vitro* and then subsequently seeded into a gelfoam matrix that was placed into intraspinal lesion cavities either alone or partially invested by a biodegradable polymeric tube. The findings of that investigation suggested that some neuritic growth had occurred within the gelfoam matrices thereby challenging the proposed neurotoxic impact of these cells.

It was unclear from that initial set of experiments, however, whether grafted microglia had a direct effect on axonal elongation. They potentially could have had a secondary influence by mobilizing other host cell or tissue types with demonstrated neurite growth-promoting properties. For example,

it is conceivable that an influx of Schwann cells or vascular elements, as well as some appropriate ECM molecules (i.e., laminin) may have occurred.

Some of the interpretational difficulty could be attributed to the biodegradable polymeric tubes that appeared to spawn a surrounding, robust inflammatory response. To control for this complication more rigorously, the present study was carried out using Gore-tex™ implants constructed of modified Teflon, polytetrafluoroethylene (PTFE). Such PTFE tubes have been successfully used in PNS regeneration studies and only elicit a minimal surrounding tissue reaction (Valentini et al., 1989; Young et al., 1984).

In view of the suggested growth-promoting effects of microglia seen in the previous experiments, another objective of the present study was to determine how co-grafts of microglia and astrocytes would affect neuritic elongation. Additionally, these experiments included PTFE tube implants that were impregnated with cultured microglia stimulated with lipopolysaccharide (LPS) prior to transplantation. Reasons for including the LPS-activated microglia group were based on the neuritic growth seen in areas of endogenous microglia/brain macrophage activation in the previous experiments, as well as the possibility that cultured microglia, although already in a macrophage state, may not be activated to secrete factors influencing cell survival and/or differentiation. In addition, it may determine whether stimulated, cultured microglia produce a neurite-inhibitory effect *in vivo*. Portions of this study have been previously summarized (Rabchevsky et al., 1994; 1995).

Methods and Materials

Isolation of Cultured Glial Cells

After several weeks of isolating microglia as described previously (see Materials and Methods, Chapter 2), 12 mM lidocaine was added to complete medium (DMEM-10% FBS) and the cultures were put on an orbital shaker set at 500 r.p.m. for 10 minutes to remove the majority of microglia from the underlying astrocyte layer (Nakajima et al., 1989). The supernatants were collected for microglial isolation, the cultures were again fed with complete medium, and the flasks were put on an orbital shaker set at 250 r.p.m. for 14 hours (37^{0}C). The floating cells were removed and the flasks once again fed with complete medium containing 5mM EDTA for 10 minutes while slowly agitating. The supernatants were aspirated and the flasks rinsed several times before DMEM containing 0.15% trypsin was added to remove adherent astrocytes. The cells were collected and an equal volume of complete medium was added to quench enzymatic activity. The astroglial suspensions were pelleted and resuspended in complete medium for impregnation of PTFE tubes. The mixed glial suspensions were made by combining resuspended microglial and astrocyte pellets. Before and after transplantation, samples of the suspensions were plated and cultured for immunocytochemical characterization.

Activation of Cultured Microglia

Microglia were isolated and grown in complete medium before stimulating them with bacterial lipopolysaccharide (LPS) which was chosen to activate microglia because it does not induce them to secrete 'neurotoxins' (Giulian et al. 1993a) yet causes them to release cytokines (Giulian et al., 1986). The chosen measure of activation was the production of IL-1 mRNA in

stimulated versus non-stimulated cultures following the protocol of Hetier et al. (1988). Confluent cultures of microglia were grown in 60 mm plastic petri dishes containing complete medium for several days. The cells were then fed with fresh medium containing 2 or 10 μg/ml LPS and exposed for 4 or 24 hrs. Then, the control and stimulated microglia were removed from the petri dishes by replacing the LPS medium with complete medium containing 12 mM lidocaine followed by vigorous agitation (500 rpm) for several minutes (Nakajima et al., 1989). Nearly all adherent microglia were removed. Supernatants were collected and placed into conical centrifuge tubes containing an equal amount of complete media before pelleting (400 x g, 10 min.). The microglia were resuspended in PBS and added to 1.5 ml Eppendorf tubes (~2 x 10^6 cells/ml) before pelleting (2000 x g, 5 min.). They were then stored at -70^{0}C until RNA extraction. The extraction of total RNA and the Northern Blot analysis were performed by Sharon Walter of Dr. Wolfgang Streit's laboratory in the University of Florida Neuroscience Department.

Impregnation of Gelfoam-PTFE Tube Implants

Polytetrafluoroethylene (PTFE) tubes (Gore Technologies; Flagstaff, AZ) measuring ~3 mm x 1.5 mm (inner diameter) were UV sterilized and filled with sterile gelfoam. The porosity of the PTFE channel walls was 30 μm and the wall thickness was ~ 500 μm. After placing the tubes upright within a sterile petri dish, a concentrated suspension of cultured glial cells (~50,000 cells/μl) was slowly injected with a sterile 22 gauge Hamilton syringe to saturate the gelfoam-filled lumen. The tubes were kept in this position for several minutes before inverting them and repeating this procedure. The saturated tubes were then placed on their sides and immersed in complete

media for subsequent incubation before transplantation (4-48 hrs.). Control PTFE implants contained gelfoam matrices soaked only in complete medium.

Impregnation of LPS-Stimulated Microglia

PTFE tubes were saturated with cultured microglia and then stimulated with LPS prior to transplantation as mentioned above. After allowing the cells to adhere to the gelfoam and the surrounding petri dish surface (>60 min.), they were incubated in complete medium containing 2 μg/ml of LPS. The cells were left at 37^0C until implantation 4-12 hrs. later at which time the LPS-containing medium was replaced with fresh complete medium.

Surgical Procedures

Control and experimental animal groups are outlined in Table 3-1. Adult female (150-300g) Sprague-Dawley rats (N=51) were anesthetized (Ketamine: Xylazine-90:10 mg/kg, I.P.) before a laminectomy was performed at vertebral T10. A midline dural incision was made for approximately 3-5 mm length. A suction micropipet was then used to create a dorsolateral cavity ~4 mm long. Hemostasis was achieved by replacement of dry gelfoam pieces into the cavity. Implants were then put into the damaged spinal cord after which the dura was sewn together with 10-0 Ethicon suture. A piece of gelfoam was laid on top of the dura before the muscles and skin were sutured. Bladders were expressed as required.

Tissue Processing

After survival times of 2, 3 or 5 weeks, the rats were overdosed with 4% chloral hydrate before transcardial perfusion with phosphate-buffered saline (PBS) containing heparin and sodium nitrate, followed by 4% paraformaldehyde in PBS (PF). For plastic semi-thin sectioning, some of the 5 week survivors were perfused with a double aldehyde solution containing 5.0% glutaraldehyde and 4.0% paraformaldehyde in 0.1 M PBS.

CATEGORIZATION OF PTFE IMPLANTS

GROUP I	GROUP II	GROUP III	GROUP IV	GROUP V
CONTROL	MICROGLIA	LPS-MICROGLIA	MIXED GLIA	ASTROCYTES
(N=13)	(N=16)	(N=8)	(N=4)	(N=5)

Table 3-1. The animals were divided into 4 experimental groups and 1 control group. The control group received PTFE tube implants containing gelfoam soaked only in culture medium. The other 4 groups each received implants containing matrices seeded with microglia and/or astrocytes derived from primary cultures of rat CNS. The LPS-microglia group consisted of seeded implants which were exposed to LPS prior to implantation in order to "activate" the grafted cells. The mixed glial implants served to characterize the effects that astroglia may have on putative growth-promoting properties of microglia. Animals were sacrificed 5 weeks after implantation and perfused with 4% paraformaldehyde. Cryosections (~10 mm) were immunohistochemically examined for cellular and neuritic profiles within and around the implants. Some animals were perfused with a dual aldehyde fixative for analysis of plastic embedded PTFE tubes (Group I, N=3; II, N=4; V, N=1).

The spinal cords which were perfused with 4% PF were immediately dissected and post-fixed in 4% PF for 30 min. before putting them in 30% sucrose/PBS for several days at 4°C. Specimens were then embedded in OCT mounting media and frozen in liquid nitrogen-cooled isopentane. They were stored at -70°C until cryosectioning (10-15 μm) and mounting them onto gelatin-coated slides that were again stored at -70°C until immunohistochemistry was performed.

The spinal cords which were perfused for plastic embedding were dissected the day after perfusion and post-fixed in the same solution for several days. The segments containing the tubes were then divided into two pieces by a transverse section midway through the implant, and the tissue blocks were osmicated, dehydrated, and embedded in Epon for transverse sections (2 μm).

Microglia isolated from mixed glial cultures were maintained either on glass coverslips or in 35 mm plastic petri dishes. Before immunostaining, cultures were washed twice with PBS and then fixed with 4% PF for 20 min., or acetone for 10 minutes.

Immunofluorescent Cytochemistry

In Situ. Before applying primary antibodies to air-dried sections on slides, the sections were incubated for 30 min. with PBS containing 3% serum from the species in which the secondary antibodies were raised. Then, primary antibodies diluted in PBS containing 1% of the same serum were applied overnight at 4°C [OX-42 (Serotec; 1:200); mouse anti-neurofilament (NaP_4-phosphorylated; 1:400); mouse anti-GFAP (Sigma; 1:400); rabbit anti-laminin, CGRP, 5-HT (Sigma; 1:500)]. After several washes with PBS, secondary antibodies in PBS were applied for 2 hours at room temperature [biotinylated anti-mouse/rabbit (Vector; 1:300; 1:500); goat anti-mouse IgG-

TRITC (Jackson; 1:400)]. If there was a tertiary antibody [streptavidin-FITC/TRITC (Vector; 1:300)], the procedure was repeated, incubating with this antibody in PBS for at least 1 hour at room temperature. Control slides were incubated with PBS/1% serum without primary antibodies.

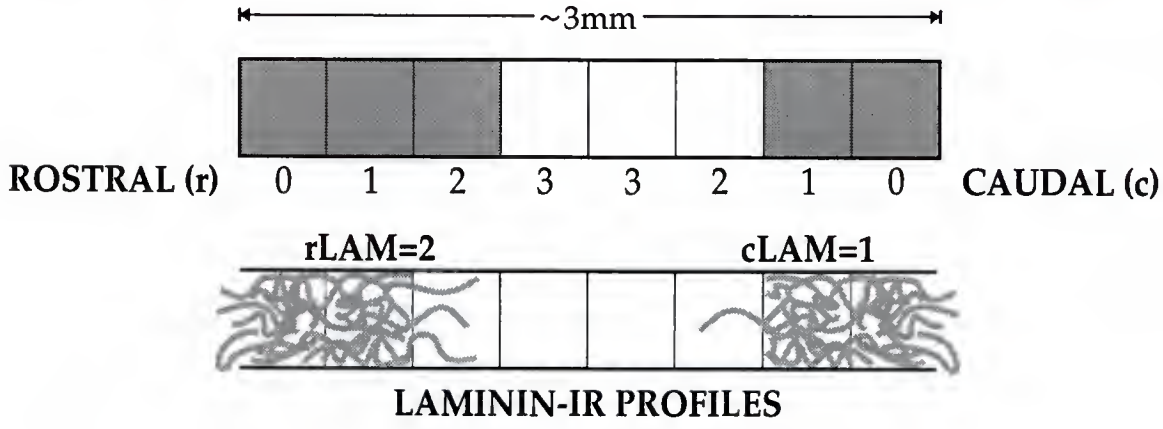
When all incubations were complete, the slides were washed several times with PBS and then coverslipped with glycerin/PBS (Citifluor; Canterbury, U.K.) mounting medium for viewing under a Zeiss microscope equipped with epifluorescent illumination (Axiophot filters: #01-UV; #09-FITC; #14-RITC). Hoechst 33342 (Sigma) nuclear dye was added to the mounting media (5μM) in order to get a better estimate of cellular profiles. Coverslips were sealed onto the slides with Cutex nail hardener (#01).

In Vitro. After fixation, the dishes/coverslips were washed with PBS before incubating the cultures with primary antibodies or GSI-B4-FITC lectin (Sigma; 10 μg/ml) in PBS for 2 hr. at room temperature. The dishes/coverslips were then washed several times with PBS and incubated with secondary antibodies in PBS for 2 hrs. The remaining procedures were done as described above.

Quantitative Analysis of Immunoreactive Profiles in Implants

To assess the content and/or infiltration of immunoreactive elements in the PTFE tubes, three slides (ea. containing ~6 cryosections) from all implants were stained with OX-42, laminin (LAM) and/or neurofilament (NF) antibodies. The tubes, which were all ~ 3 mm in length, were examined microscopically and divided into rostral (R) and caudal (C) segments. Then, the R and C portions of the tubes were again divided into 4 ordinal modes that were used to rank the content of immunoreactive profiles seen at both ends of the tubes. An example of this method for ordinal ranking is

EXAMPLE OF METHOD TO ORDINALLY RANK IMMUNOHISTOCHEMICAL PROFILES IN PTFE TUBES



"Kruskal-Wallis" anova Test by Ranks

VARIABLE	SCORE	SIGNIFICANCE
rOX-42	H (4,N=579) = 110.4606	P < 0.0001
cOX-42	H (4,N=579) = 219.4768	P < 0.0001
rLAM	H (4,N=567) = 85.99577	P < 0.0001
cLAM	H (4,N=567) = 220.6777	P < 0.0001
rNF	H (4,N=559) = 95.73747	P < 0.0001
cNF	H (4,N=559) = 180.9473	P < 0.0001

* Test does not support the "Null Hypothesis" that these experimental groups are from the same population (Statistica by StatSoft).

Fig. 3-1. These schematics represent an example of the method used to assign ordinal rankings to the immunohistochemical profiles observed in each stained cryosection from the PTFE implants. The ranks were determined by establishing the existence of at least three immunoreactive profiles (r and c) seen within an ordinal mode (0-3) for each stain. After compiling all the ordinal data, shown schematically in the upper-most figure, a "Kruskal-Wallis" a nova test by ranks was performed to establish whether these data were from the same experimental populations.

illustrated in Fig. 3-1. After the ordinal data were collected from all R and C segments of each implant, the Kruskal-Wallis a nova test by ranks determined that the independent samples (i.e., rOX-42, cOX-42, rLAM, cLAM, rNF, cNF) were from different populations (Fig. 3-1). After summarizing the ordinal data for each variable within the implants, the Mann-Whitney U test was performed to determine the significant differences among these various groups.

Results

General Features of PTFE Implants

As noted earlier, in our previous study of intraspinal microglial implants (Chapter 2) we had used a biodegradable polymer tube as a way to provide a more structured orientation of the biomatrix (i.e., cell-seeded gelfoam). A complication of those tubes was that they induced a robust inflammation probably as a consequence of degradation; this was not the case with the PTFE tubes that were used in the present study. Neither at 2, 3 or 5 weeks post-implantation was there evidence of either profuse cellular accumulations along the exterior surface of these tubes (Fig. 3-2A,B) or linearly oriented neurofilament immunoreactive (NF-IR) profiles coursing alongside (see Chapter 2). While some mononuclear cells were seen in this region, the density of these cells did not appear to be strikingly greater than that of more distant host neuropil. Neurons in the immediate vicinity of these implants appeared unaffected by the presence of this prosthetic material (Fig. 3-2B), although positive staining for phosphorylated neurofilament indicated some form of perturbation (Fig. 3-2C). Ramified microglia were

Fig. 3-2. Cytological features of host tissue surrounding the PTFE tube implants. These photomicrographs demonstrate the cellular elements immediately surrounding the implants after 5 weeks. A,B. Nissl stained preparations show the relative lack of inflammation induced by the PTFE implants. A. Microglia-impregnated PTFE implant seen in longitudinal section demonstrating an example of the variability in the impregnation of gelfoam matrices (X40). B. At higher magnification note that despite the presence of some inflammatory cells along edges of the tube, viable motoneurons are seen immediately adjacent to the wall (X120). C. Higher magnification of neurons immunofluorescently stained with a neurofilament antibody (RITC) found immediately adjacent to the tube walls (marked by arrowheads) (X320). D. Same field of view as B seen through an FITC filter to demonstrate perineuronal positioning of OX-42-IR microglia, some ensheathing apparently healthy neurons (arrows).

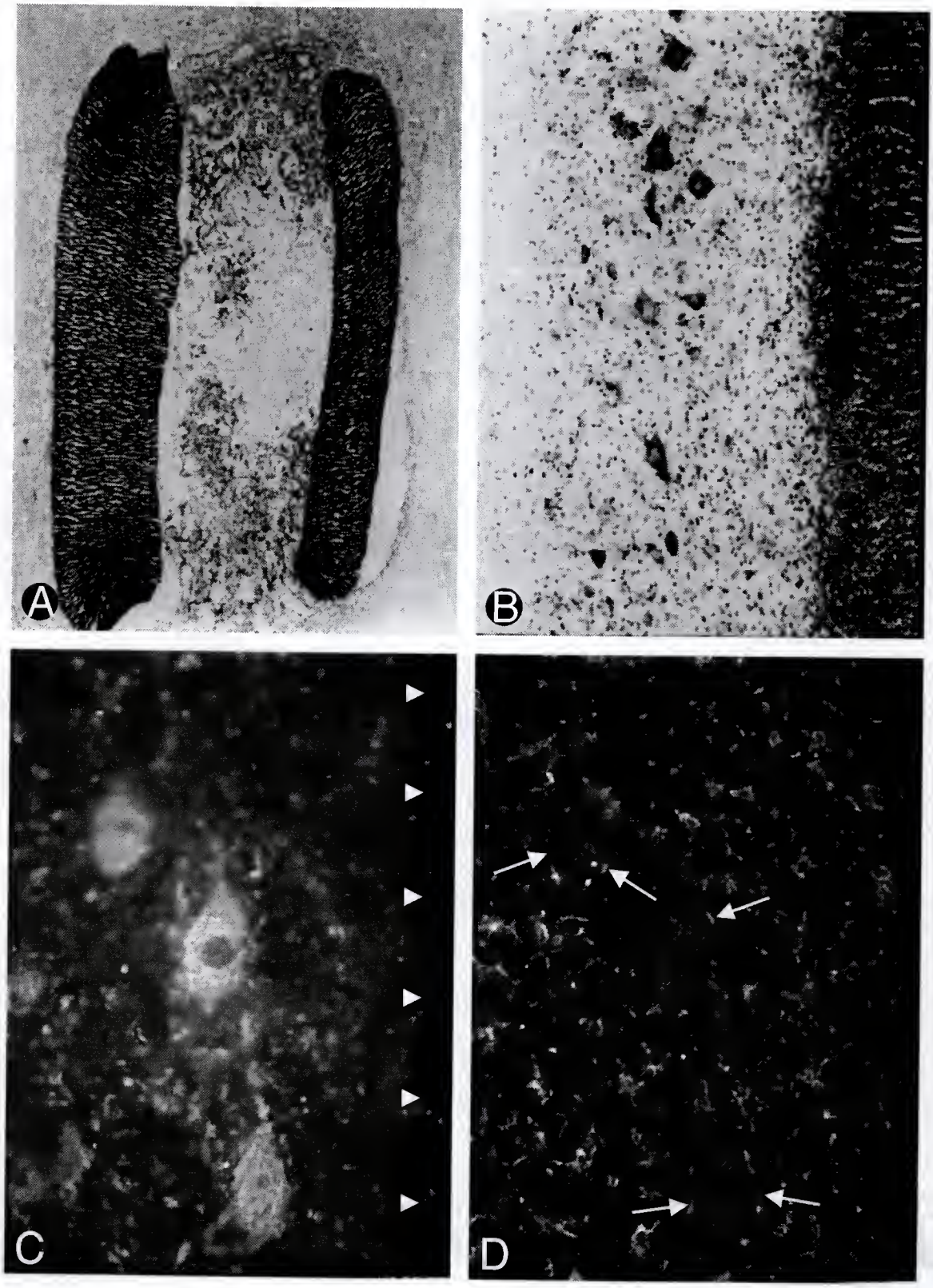

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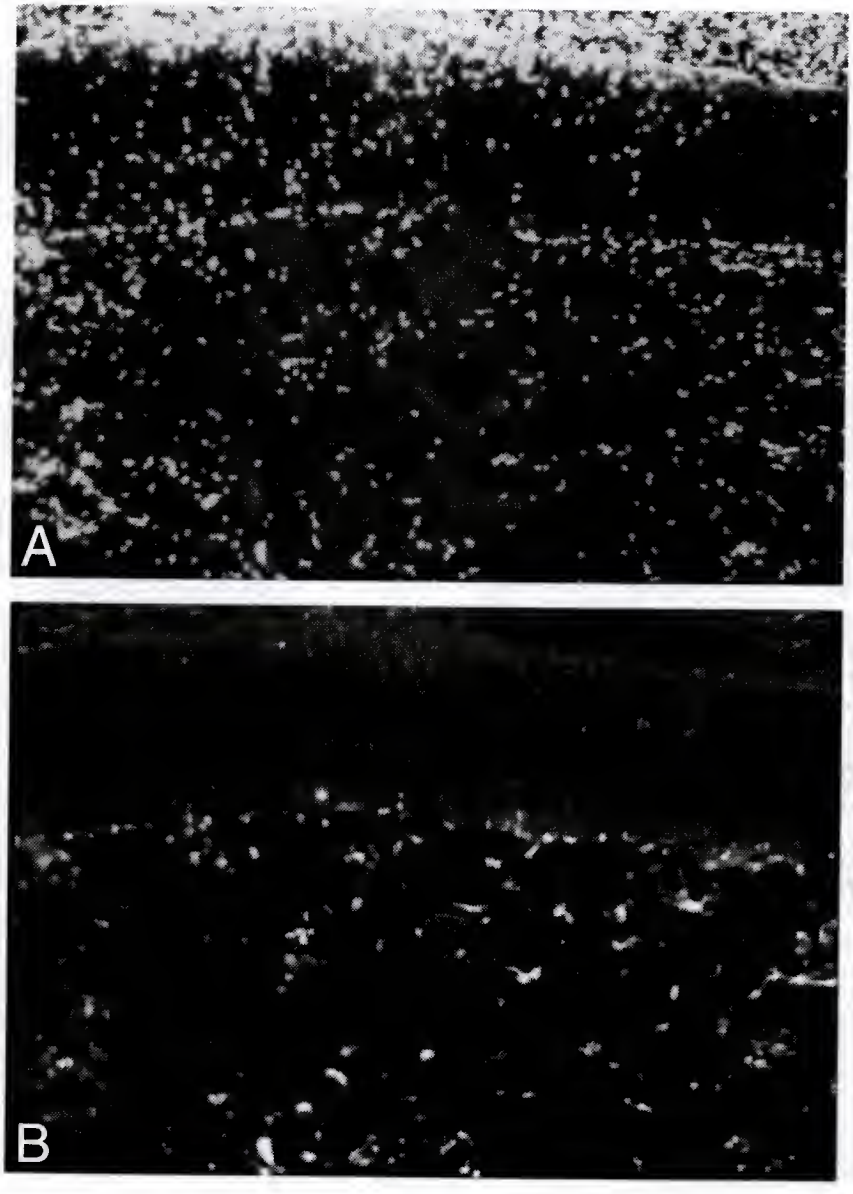
Fig. 3-3. Characterization of PTFE tubes containing gelfoam saturated with cultured microglia prelableled with DiI-ac-LDL, 3 weeks post-implantation. A,B are the same field of view seen through UV and RITC fluorescent filters, respectively, showing the distribution of cellular elements in these implants. The top of each photograph represents the PTFE walls juxtaposed to host spinal cord tissue while the gelfoam core is seen in the lower half of each frame. A. Nuclear profile (Hoechst 33342) demonstrates the distribution of cells in the matrix and that many cellular elements entered the wall channels, especially from the exterior surface (X80). B. Scattered DiI-ac-LDL-labeled microglia are seen throughout the matrix. Some elements in the Gelfoam were not labeled, suggesting that these may be derived from the host. Note that while labeled cells do line the interior surfaces of the tube walls, few were seen entering the porous channels to exit the matrix.


A
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found in the spinal gray matter surrounding these cells (Fig. 3-2D), and while some appeared activated (i.e., hypertrophied) their density and distribution did not appear strikingly different from normal parenchyma.

Because of the 30 μm porosity of the tube walls, many cellular elements from the surrounding spinal cord were observed coursing through the walls toward the abluminal surface (Fig. 3-3A). To determine whether any of these migrating cells were grafted cells, some PTFE tubes contained microglia that were prelabeled with DiI-ac-LDL (N=2). While these implants demonstrated many cells traversing the tube walls after three weeks, very few showed positive DiI-ac-LDL labeling (Fig. 3-3B).

Many of the inwardly migrating cells were microglia/brain macrophages as defined by OX-42-IR (Fig. 3-4A). While a detailed characterization of the other infiltrating cells was not performed, it was found that none of the elements within the channel walls exhibited GFAP-IR. Some regions of intense laminin-IR were seen along the external walls of the PTFE tubes, and thin extensions of this ECM molecule were often observed within the walls *per se* (Fig. 3-4B). Interestingly, there were also instances in which NF-IR profiles were seen entering these channel walls, many times paralleling the laminin-IR elements (not shown).

Cellular and Neuritic Composition of Control PTFE Implants

While there was minor surrounding inflammation elicited by PTFE, the tubes containing cell-free gelfoam soaked in culture medium still demonstrated an infiltration of host cellular elements after 5 weeks (Figs. 3-5A, 3-6A). The amount of infiltration and the preferential rostral or caudal entrance into these tubes varied from animal to animal, and after dissection no correlation could be made with regard to the tube placement and the

Fig 3-4. High magnifications of the porous PTFE walls demonstrating the infiltration of cellular elements five weeks after implantation. These photographs represent adjacent sections showing that while many of the cells coursing through the channels of the tube walls are OX-42-IR brain macrophages (A), there was also the presence of laminin-IR profiles within the lacunae (B). Note the similar distribution of OX-42-IR and laminin-IR along the outer walls of the tube.

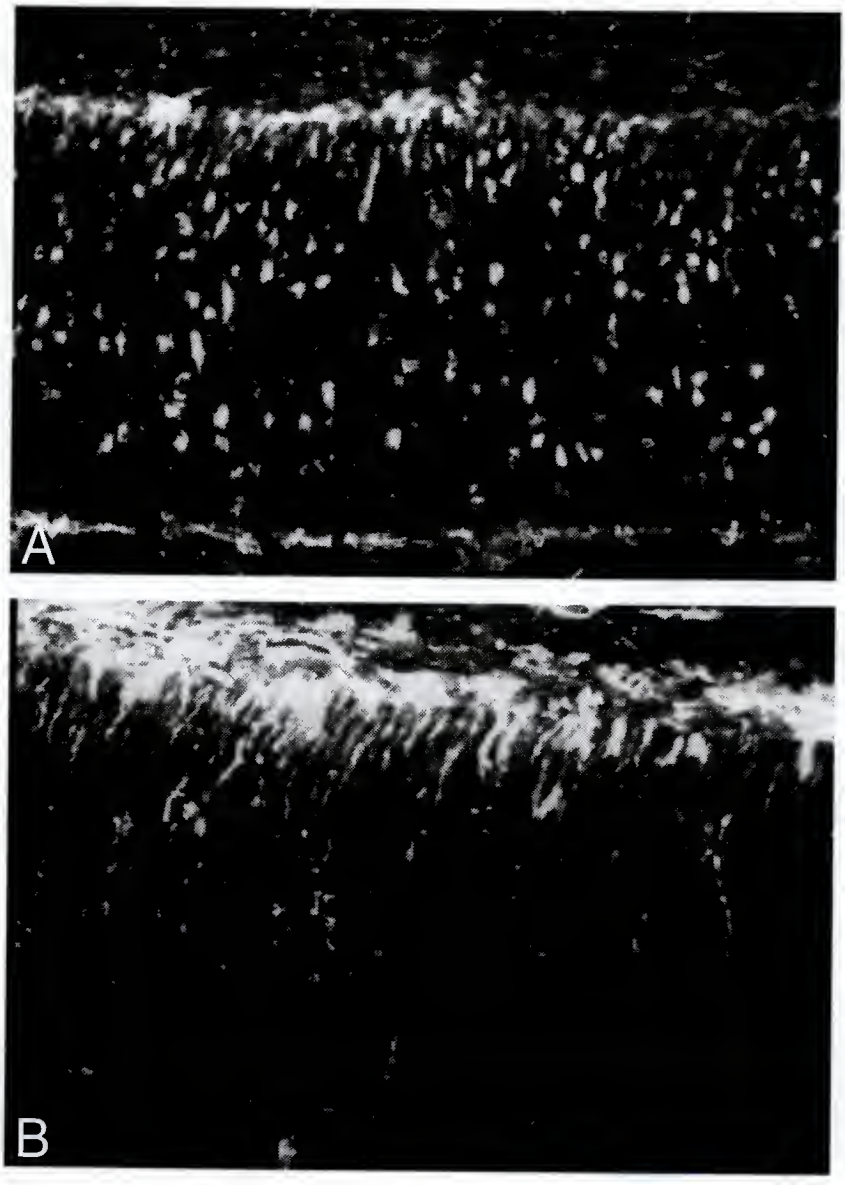
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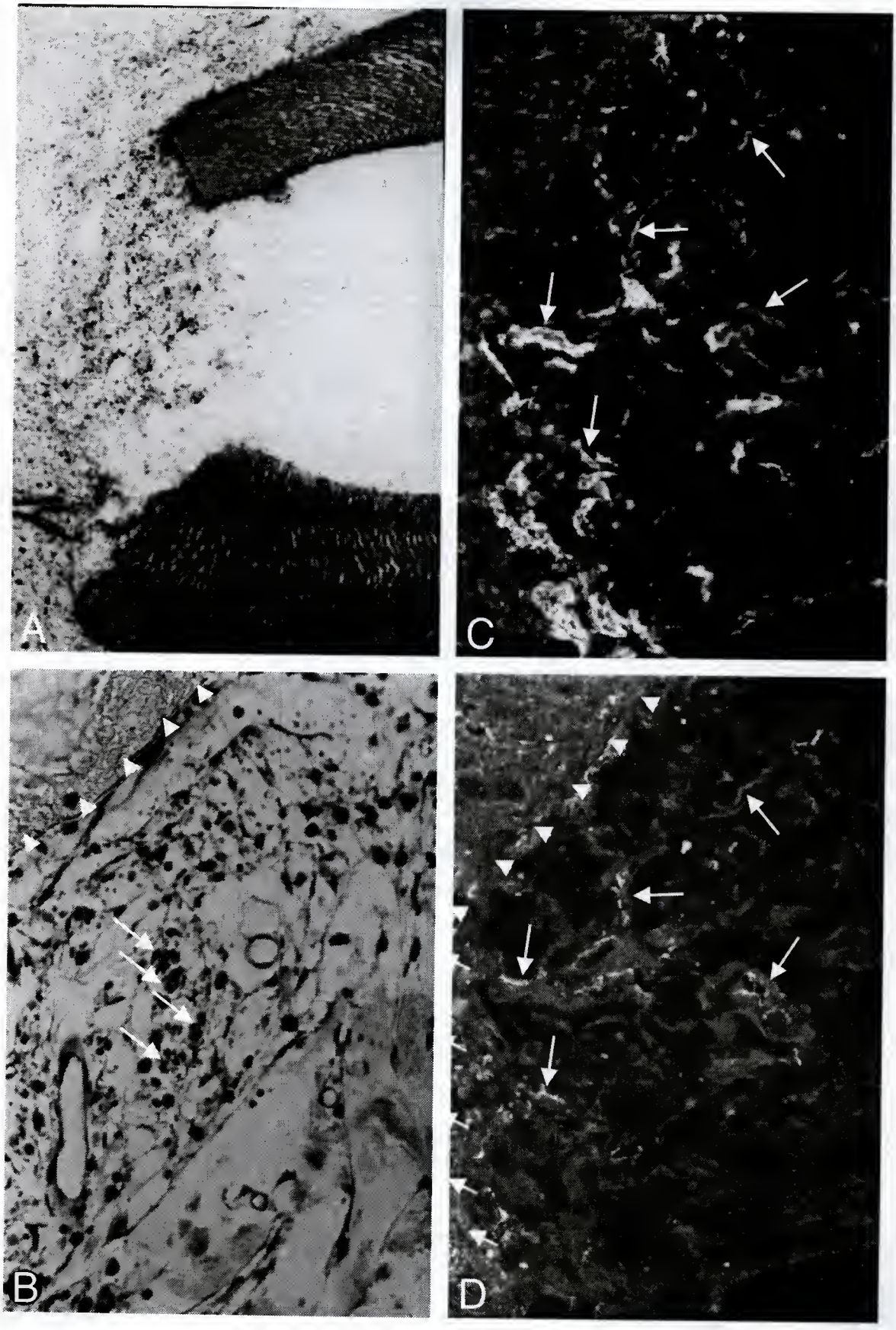
Fig. 3-5. Sections through control PTFE implants after 5 weeks. A. Nissl stain of a longitudinal, horizontal section through an implant showing an example of only slight infiltration of host cellular elements beyond the gelfoam-host border (X80). The host spinal cord is to the left side of the photograph, demonstrated by the central canal seen at the bottom left. B. Transverse, plastic section (2 μm) through a control PTFE implant (arrowheads delineate inner wall) demonstrates vascularization close to the edges of the lumen, accompanied by occasional Schwann cell-myelinated axons (arrows) in the presence of mononuclear phagocytes and other cells (X320). C,D are higher magnifications of the graft-host border in A showing the same field of view seen through different fluorescent filters to demonstrate the coexistence (arrows) of infiltrating OX-42-IR cells (C) and neuritic ingrowth (D); arrowheads demarcate the graft-host border (X120).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incidence of robust cellular infiltration. Semi-thin, plastic sections of the control PTFE tubes indicated that by 5 weeks some of the gelfoam matrix had been degraded. At this time, the interior of the PTFE tubes contained numerous mononuclear phagocytes and connective tissue elements that formed a loose trabecular lattice that appeared to provide a supporting matrix for the infiltrating cells (Fig. 3-5B). However, the increased cellularity and accompanying vascularization of these control implants was predominantly restricted to the inner walls of the PTFE tubes (Fig. 3-5B).

Immunohistochemical staining of these tubes showed prominent OX-42-IR presented by the cellular infiltrates (Figs. 3-5C, 3-6B). Staining for neurofilament revealed a modest ingrowth of neuritic profiles, the distribution of which was in register with OX-42-IR. A modest degree of laminin-IR also was noted in some tubes that coincided with OX-42-IR (Figs. 3-6B,C). The majority of laminin-IR, however, was seen at the interface between host spinal gray/white matter and the gelfoam within the walls of the tube. This region also exhibited a wall of GFAP-IR processes that to some extent was in register with the laminin-IR in the host tissue (Figs. 3-6C,D).

A correlate of the NF-IR elements, noted above, was observed in semi-thin plastic sections. Myelinated axons were seen occurring as small bundles having a rather random distribution within the tube lumen (Fig. 3-5B) but never in cell-free regions. At higher magnification (not illustrated), these myelinated sheaths were seen enveloped in some cases by cytoplasm that occasionally contained a nuclear profile as is characteristic of axon-Schwann cell relationships in peripheral nerves.

Microglia-Impregnated PTFE Implants

At 2 and 5 weeks post-implantation, the cell-seeded tubes displayed a rather dense OX-42-IR at the rostral and caudal parts of the matrices

Fig. 3-6. Sections through a control PTFE tube which demonstrated robust cellular infiltration seen 5 weeks post-implantation. The top of each photograph represents the graft (gelfoam)-host border. A,B and C,D represent same fields of view, respectively, seen through AMCA (A), FITC (B,D) or RITC (C) fluorescent filters. A. Prominent infiltration of endogenous cells was seen in some control implants, most of which were OX-42-IR (B) (X80). C. Adjacent section to B demonstrating laminin-IR in register with infiltrating brain macrophages and other cellular elements (X80). Note that the majority of staining, however, is confined to the graft-host border and the host spinal cord. D. In the presence of penetrating laminin-IR elements, no GFAP-IR cells were ever seen in any of the control implants (arrows indicate astroglial lining restricted to the host issue).

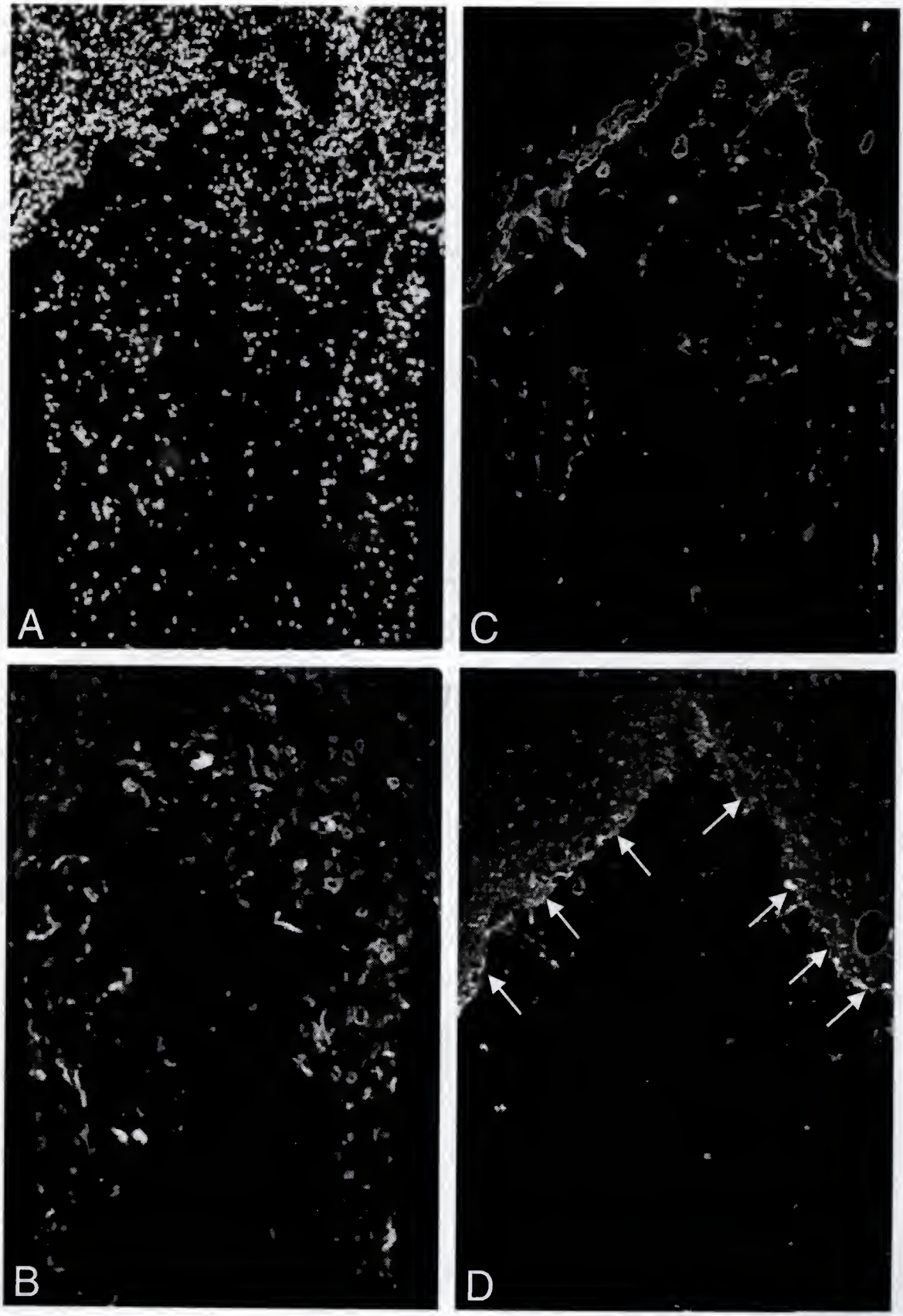
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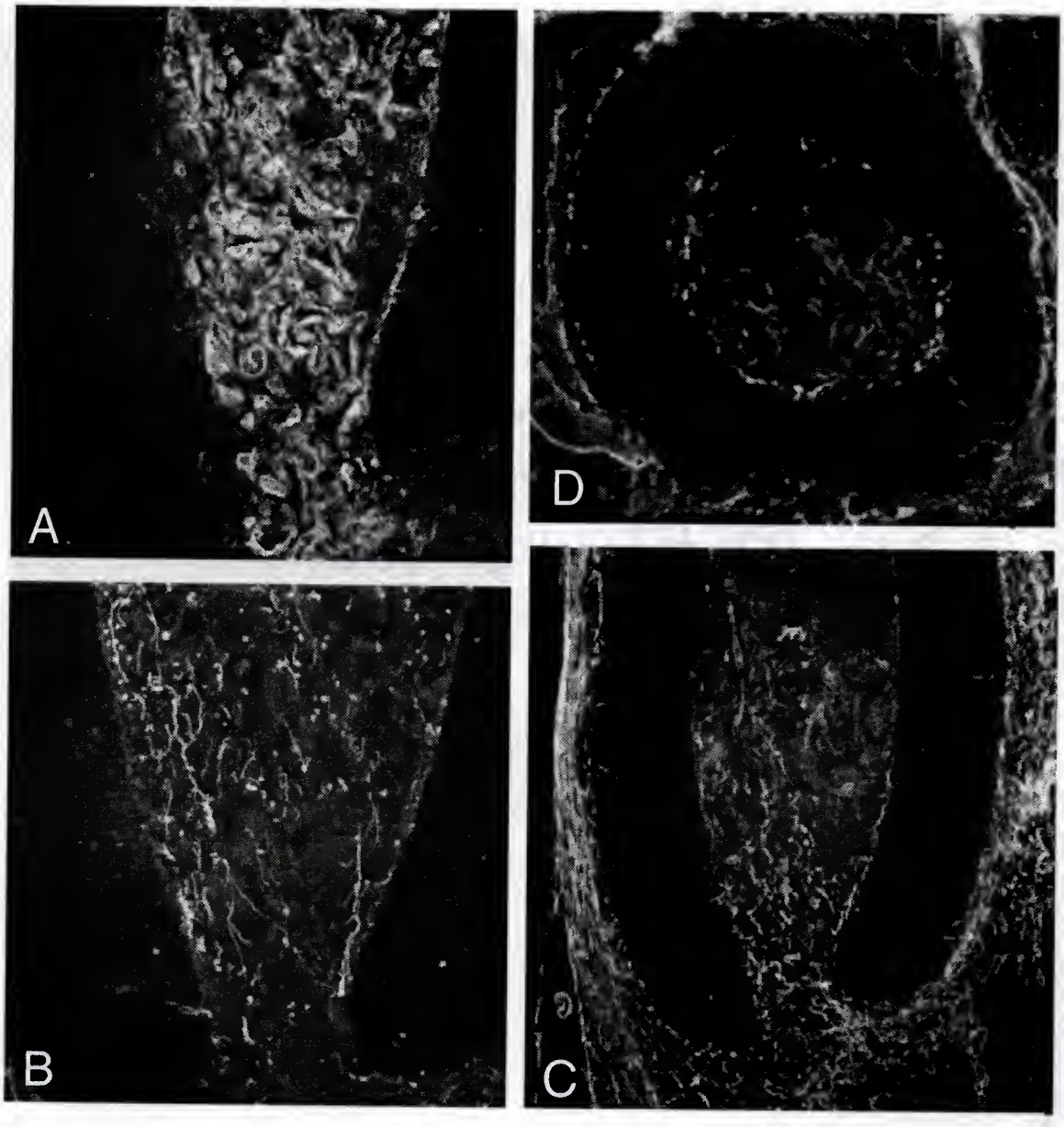
Fig. 3-7. Sections through PTFE implants impregnated with microglia. A-C represent sections through one implant examined 2 weeks post-transplantation. The lateral portions of the photographs represent the walls of the tube. A. OX-42-IR is demonstrated throughout the matrix, and there is penetration of neurofilament-IR processes (B) into the microglial environment (X60). Penetration of laminin-IR elements (C) also paralleled the neuritic ingrowth (X40). Laminin-IR was even more apparent throughout the matrices after 5 weeks, seen in a cross section (D) of a microglia-impregnated tube (X40). Note that the entire implant was invested by an intense laminin-IR lining.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(Figs. 3-7A, 3-8A). While most tubes had a relatively homogeneous distribution of OX-42-IR cells, the concentration of such cells in the center of some tubes was considerably reduced due to difficulties in obtaining complete cell-seeding routinely (Fig. 3-2A). Anti-neurofilament staining demonstrated neurites with linear or branched trajectories distributed within the OX-42-IR domains (Figs. 3-7A,B; 3-9A,B). Qualitatively, the degree of neuritic ingrowth appeared to be substantially greater than that noted in control tubes, and neurofilament-IR processes were distributed throughout the matrices in all orientations.

By 5 weeks after implantation, transverse sections of the spinal cord showed that the area occupied by the PTFE tubes essentially represented a hemisection lesion (Figs. 3-7D, 3-10A). The entire tube was invested by a laminin-IR lining that was in register with GFAP-IR. However, there was a cellular boundary between the exterior tube surface and the astrocytic processes surrounding it that was highly OX-42-IR (Fig. 3-4A). In contrast to control tubes (see above), a greater degree of laminin-IR also was seen within the cell-seeded matrices (Figs. 3-7C, 3-8B, 3-9D). Inside the tubes, this ECM molecule was distributed along the abluminal surface, but this did not coincide with any GFAP-IR as such was undetectable in the matrices (Fig. 3-8C). While there were examples of colocalization, the distribution of laminin-IR did not appear to be in consistent register with neuritic ingrowth (compare Figs. 3-7B,C to 3-9C,D).

Plastic sections demonstrated a greater cellularity than seen in the control implants, most notably within the core of the gelfoam (Fig. 3-10A). In addition to mononuclear phagocytes, there were numerous multinucleated giant cells in these cell-seeded tubes (Figs. 3-10B,C), along with vascular and connective tissue elements that appeared to be embedded in a collagenous

Fig. 3-8. Longitudinal, horizontal sections through microglia-impregnated PTFE tubes studied at 5 weeks post-transplantation. The extreme top and bottom of the photographs represent the tube walls and the host-graft (gelfoam) border is to the left A. Microglia-impregnated gelfoam was inundated with OX-42-IR profiles (X80). However, there were many other cellular elements in these matrices that were not microglia/brain macrophages, as revealed by cells (Hoechst 33342) that were not OX-42-IR (not shown). B. Adjacent section to A demonstrates that unlike control PTFE implants, these gelfoam matrices were filled with laminin-IR profiles, many of which appeared to be associated with microvascular elements. C. Adjacent section to B demonstrating the lack of any GFAP-IR cells in the matrix but the presence of an astroglial lining surrounding the implant (arrows).

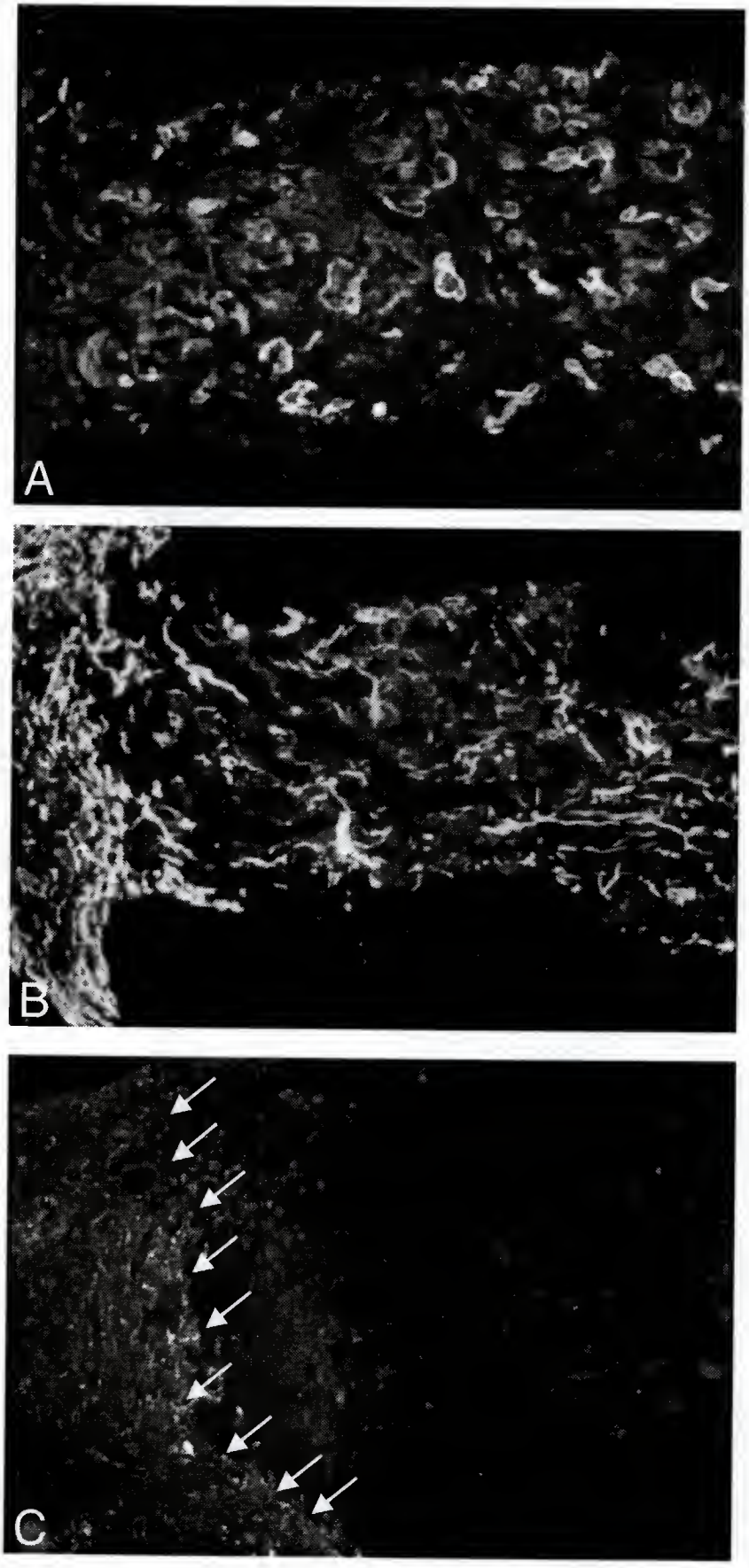
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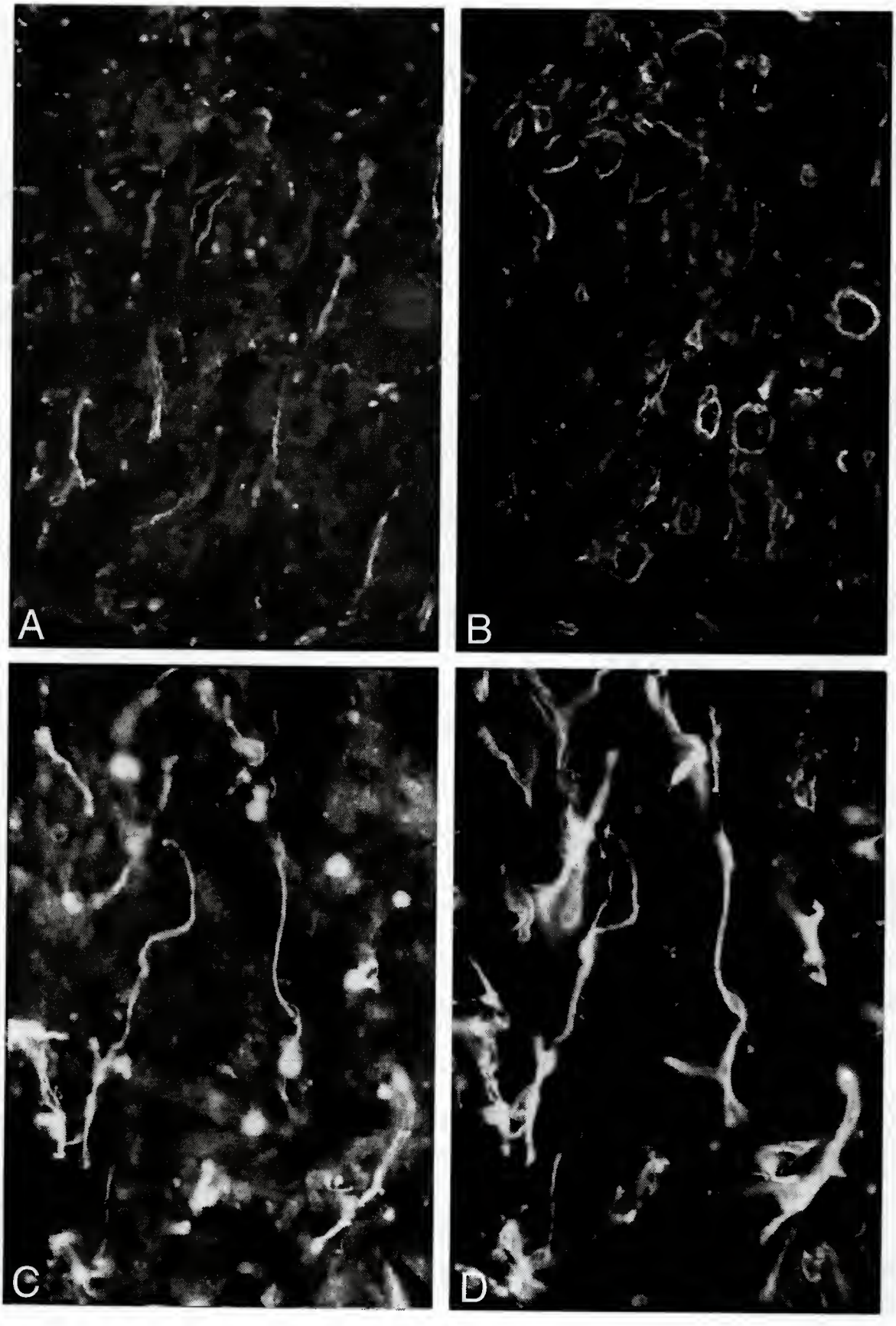
Fig. 3-9. A,B and C,D represent the same fields of view, respectively, seen through RITC (A,C) and FITC (B,D) fluorescent filters. Higher magnification of a microglia-saturated gelfoam matrix (Fig. 3-8A) stained with neurofilament antibody (A) shows neuritic processes coursing through the OX-42-IR microenvironment (B) (X320). C and D demonstrate the occasional colocalization of neuritic processes (C) with laminin-IR elements (D) (X320).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ground substance (Fig. 3-10B). As in control implants, Schwann cell myelinated axons also were seen in plastic sections throughout these cell-seeded PTFE tubes. These fibers were randomly distributed and did not appear to be consistently related to any cell or tissue type present in these grafts (Fig. 3-10B).

PTFE Implants Containing LPS-Activated Microglia

Some PTFE tubes saturated with cultured microglia were incubated in complete medium containing 2μg/ml LPS for 4-12 hours before implantation. This incubation period was based on the level of IL-1 mRNA expression which is a standard measure of microglial activation *in vitro* (Fig 3-11A). In addition, inspection of the petri dishes containing the saturated tubes revealed morphological alterations of microglia adhering to the dishes. This correlated with LPS-induced levels of IL-1 mRNA expression (Figs. 3-11B,C).

Implants saturated with LPS-stimulated microglia displayed similar immunohistochemical profiles as the unstimulated microglial tubes after 5 weeks. Thus, a comparable pattern of laminin-IR and NF-IR distribution was observed in OX-42-IR rich areas of the gelfoam (Figs. 3-12A,B). However, a notable difference appeared to be a more pronounced infiltration of various host cellular elements into the LPS-stimulated microglial matrices.

Astrocyte-Seeded PTFE Implants

There were actually two subsets of implants within the astrocyte implant group; those that were impregnated with a mixed population of cultured microglia and astrocytes, and those that were impregnated with an enriched suspension of astrocytes. Cultures of the transplanted cells were made with the remaining suspensions after surgery in order to immunocytochemically identify the cellular constituents. While the mixed

Fig. 3-10. Transverse, plastic sections (2 μm) through PTFE tubes impregnated with microglia 5 weeks after implantation. A. Prominent cellularity is demonstrated within these implants compared to controls (Fig. 3-5B), especially within the core of the gelfoam (X10). B and C demonstrate the increased concentration of macrophages and blood vessels within these matrices (X320). While there were groups of Schwann cell-myelinated axons found within regions of macrophage accumulation (arrows), most areas contained large, multinucleated cells (arrowheads) and microvasculature associated with mononuclear cells in the absence of myelinated axons (C).

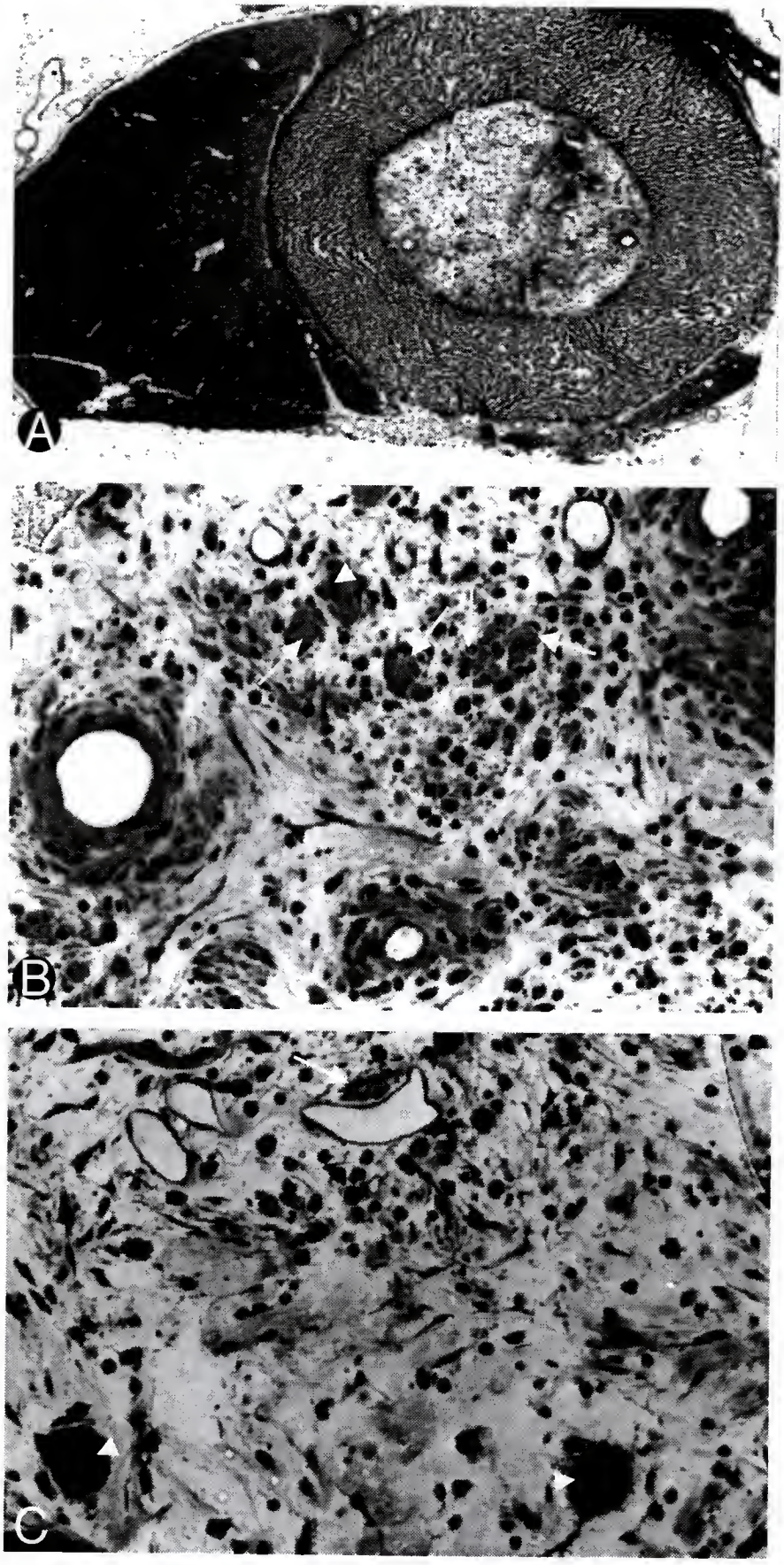
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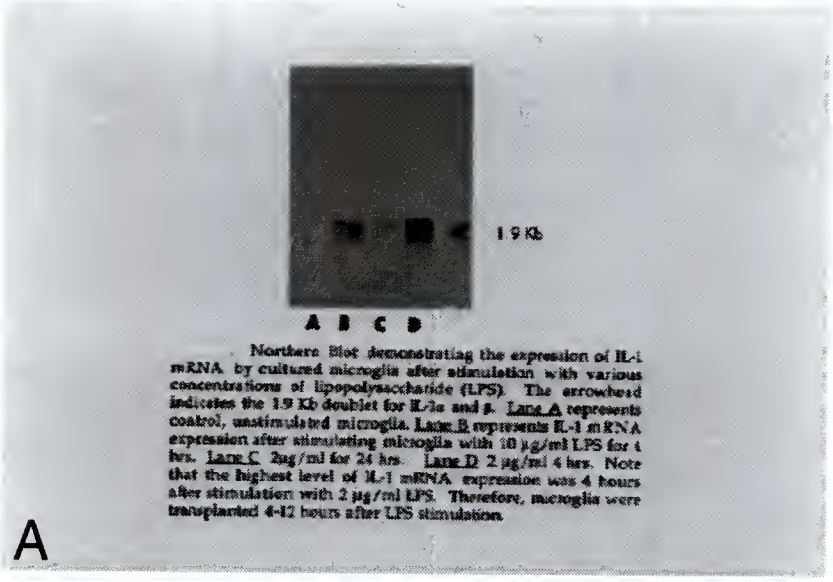
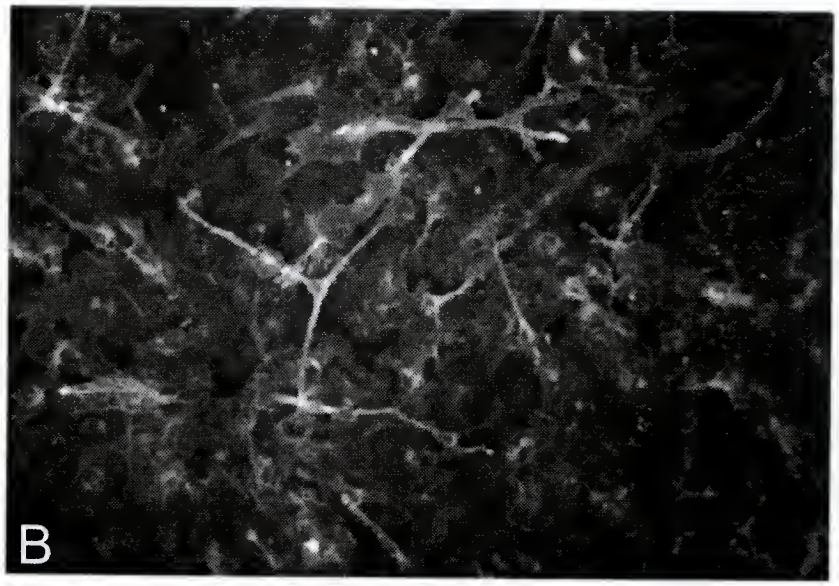
Fig. 3-11. Characterization of cultured microglia stimulated with lipopolysaccharide (LPS). A. Northern blot demonstrating the expression of IL-1 mRNA by cultured microglia after stimulation with various concentrations of LPS. B. The lectin-conjugate GSI-B_4-FITC was used to demonstrate phenotypic changes in microglia that were cultured in the presence of 2 μg/ml LPS for 4 hours (X200). Note the elongated, highly branched morphology of these activated microglia as compared to unstimulated cultured microglia (see Fig. 4-2). C. After 24 hours of exposure to LPS, microglia had retracted their processes and assumed a more flattened, ameboid morphology. This may correlate with their decreased IL-1 mRNA expression (A, lane C) (X320). Note: Sharon Walter performed the RNA extraction and created the Northern Blot shown.



Northern Blot demonstrating the expression of IL-1 mRNA by cultured microglia after stimulation with various concentrations of lipopolysaccharide (LPS). The arrowhead indicates the 1.9 Kb doublet for IL-1α and β. Lane A represents control, unstimulated microglia. Lane B represents IL-1 mRNA expression after stimulating microglia with 10 μg/ml LPS for 4 hrs. Lane C 2ug/ml for 24 hrs. Lane D 2 μg/ml 4 hrs. Note that the highest level of IL-1 mRNA expression was 4 hours after stimulation with 2 μg/ml LPS. Therefore, microglia were transplanted 4-12 hours after LPS stimulation.

A



B

C

glial population contained ~60% microglia (GSI-B4), astrocyte enriched populations had >80% GFAP-IR cells (Fig. 3-13A-D).

The mixed glial implants demonstrated a robust ingrowth of neurites and laminin-IR elements similar to microglial grafts. In addition, OX-42-IR seemed equally as robust as in the microglia-impregnated implants. Interestingly, however, no GFAP-IR cells were detected within these matrices.

In light of the *in vitro* characterizations of the graft suspensions (Fig 3-13C,D), a more unexpected finding was the scant GFAP-IR cells seen within the enriched astrocyte implants (Fig. 3-14A). Those cells that did express GFAP were almost exclusively associated with laminin-IR elements, some of which did not resemble characteristic microvascular constituents (Figs 3-14B,C). However, many of the GFAP-IR profiles did colocalize with laminin-IR vessels, especially those near or entering the wall channels (Figs. 3-15A,B). In many regions devoid of GFAP-IR cells, there were areas in the gelfoam that contained numerous OX-42-IR cells (Fig. 3-15C). Despite the lack of many GFAP-IR cells in these implants, plastic sections confirmed that they contained numerous islands of astroglial cells extending processes throughout the gelfoam matrix, and few neurites were seen in these grafts (Figs. 3-16B,C).

Interestingly, most of the neuritic growth observed in astrocyte grafts was into the caudal end of the PTFE tubes. Like all other cell-impregnated PTFE tubes, the astrocyte implants were penetrated by microvasculature and other elements, most notably brain macrophages (Figs. 3-16A-C). However, few Schwann cells were seen in plastic sections, and those observed were myelinating axons in astrocyte-free regions of gelfoam.

Fig. 3-12. Microglia-impregnated PTFE implants 5 weeks after activation with LPS and subsequent grafting. The top and bottom of the photographs represent the lateral margins of the gelfoam matrix, and the host-graft (gelfoam) border is to the left. A. Sections through the lumens of microglial implants exposed to LPS before transplantation demonstrated similar penetration of laminin-IR profiles to that seen in non-stimulated microglial implants (X80). B. Neurofilament staining of an adjacent section demonstrates that neuritic penetration was also coincident with the appearance of laminin-IR elements. However, there seemed to be an overall increase in the cellularity within these implants compared to non-activated microglial grafts, as evidenced by the more compact aggregation of nuclear profiles (Hoechst 33342) and OX-42-IR cells in regions of these matrices.

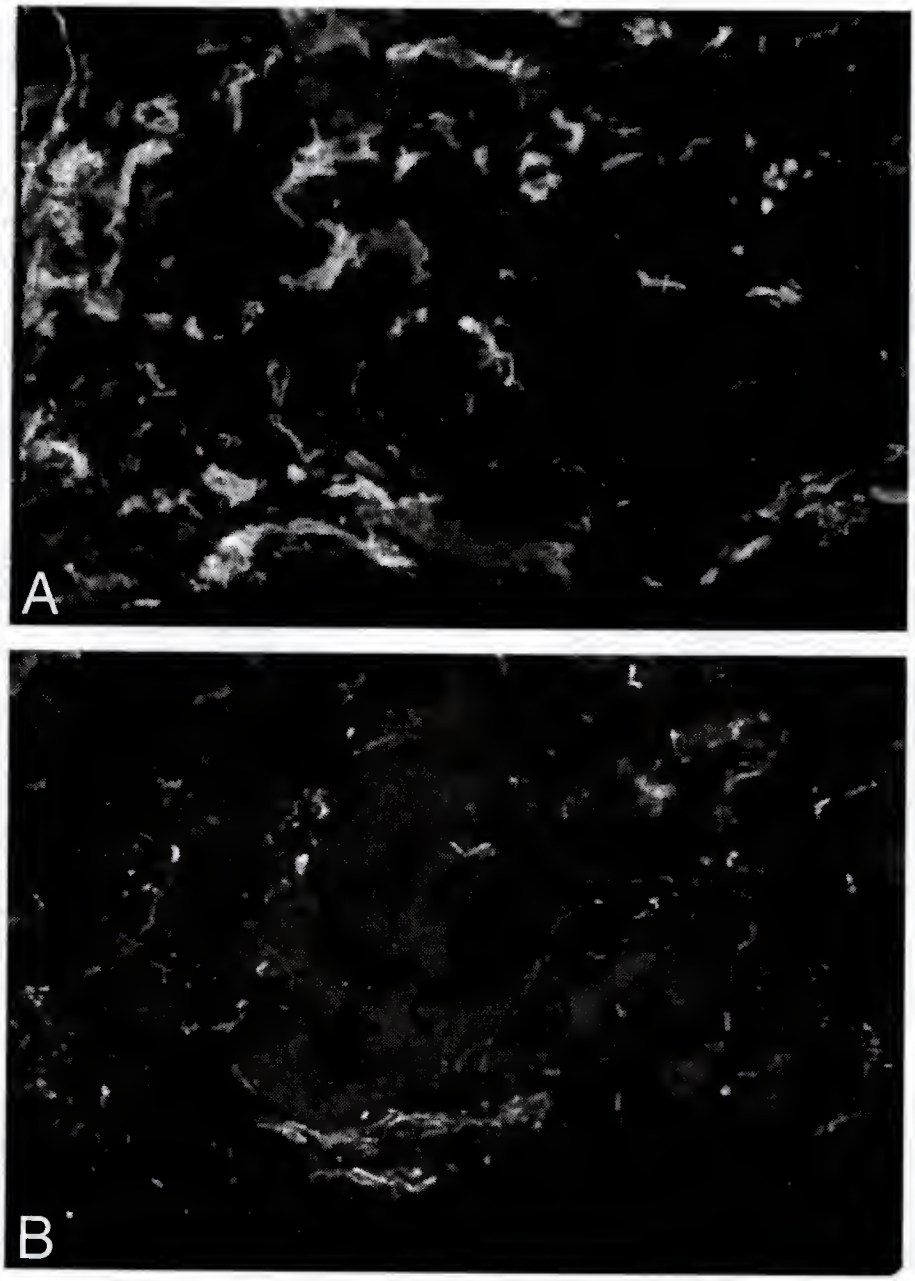
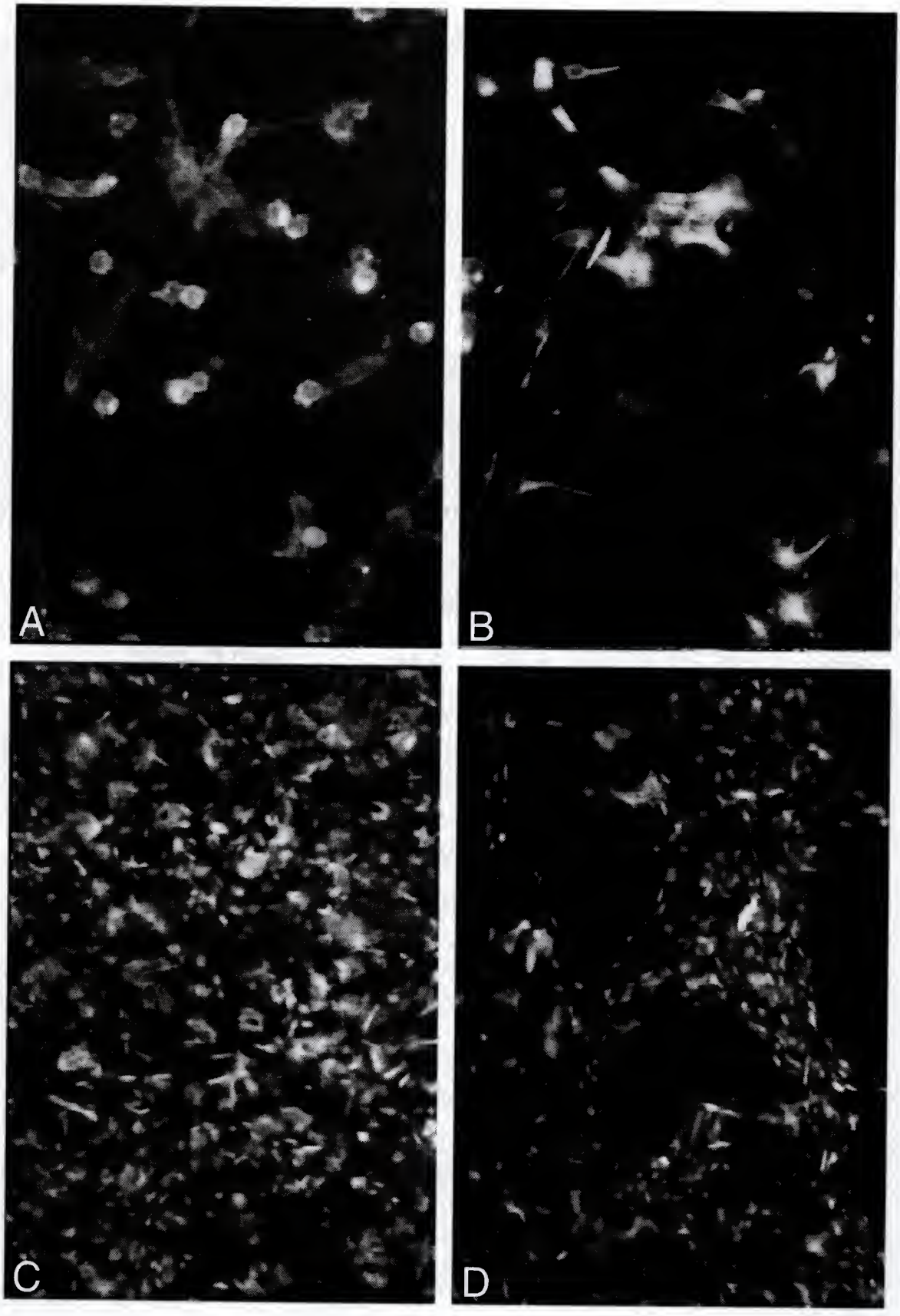

A
B

Fig. 3-13. Astrocyte and mixed glial transplant characterizations. A and B represent identical fields of a mixed glial suspension cultured and double-stained for GSI-B4 (FITC) and GFAP (RITC), respectively (X320). C and D represent an enriched astrocyte suspension cultured and stained for GFAP (RITC). While C,D show high levels of GFAP expression, some regions of the culture contained neither GFAP-IR nor lectin-positive cells (D). Interestingly, the nuclei (Hoechst 33342) of these non-labeled cells very much resembled neighboring GFAP-IR cells (not shown).


A
B
C
D

Quantitative Evaluation of PTFE Control and Experimental Implants

An effort was made to put the qualitative observations described above into a quantitative context. As noted in Material and Methods, this entailed compiling the ordinal measurements of immunoreactive (IR) profiles seen within the rostral (R) and caudal (C) segments of cryosections through each PTFE tube implant after 5 weeks (Fig. 3-1). In brief, after each implant was immunohistochemically stained with OX-42, neurofilament (NF) or laminin (LAM) antibodies, an ordinal rank (0-3) was given to the amount of immunoreactivity seen in R and C portions of the matrices based on the observation of at least 3 immunoreactive profiles within an ordinal mode (0-3). A summary of all the data is schematically represented in Fig. 3-17. Non-parametric statistics were then used to determine whether there were significant differences in the immunohistochemical profiles among the various implant groups (Fig. 3-19).

In summary, the control tube implants demonstrated the presence of OX-42-IR cells accompanied by neurofilament- and laminin-IR profiles that were all significantly less than those seen in the cell-seeded implants (Figs. 3-18,19). This is illustrated in Fig. 3-20 showing that the infiltrating OX-42-IR cells were accompanied by neurofilament- and laminin-IR profiles, especially prominent in the rostral end of the implant. For comparison, Figs. 3-19,20 illustrate that the R and C segments of the microglia-impregnated tubes contained significantly more immunoreactive profiles throughout their matrices compared to the control tubes after 5 weeks. However, there were no significant differences between the LPS-stimulated versus non-stimulated microglial implants, except in the rankings for caudal laminin-IR (Fig. 3-19).

Interestingly, the mixed glial implants demonstrated the highest ordinal rankings with the least variances of any group, and were found to

Fig. 3-14. PTFE implants impregnated with cultured astrocytes 5 weeks after transplantation. The top and bottom of A represent the lateral walls of the implant (host tissue not seen). However, the lateral walls of the implant are located to the left and right in B,C. A. Longitudinal, horizontal section through an astrocyte-seeded PTFE tube stained for GFAP demonstrates the unexpectedly scarce detection of GFAP-IR cells within these implants, and the apparent emigration of some these cells through the channels of the wall (arrows; X80). B and C both represent the same field of view seen through FITC and RITC fluorescent filters, respectively, in order to demonstrate the coexistence (arrows) of GFAP-IR elements (B) almost exclusively with laminin-IR profiles (C) that, in many instances, did not resembled microvasculature (X120).

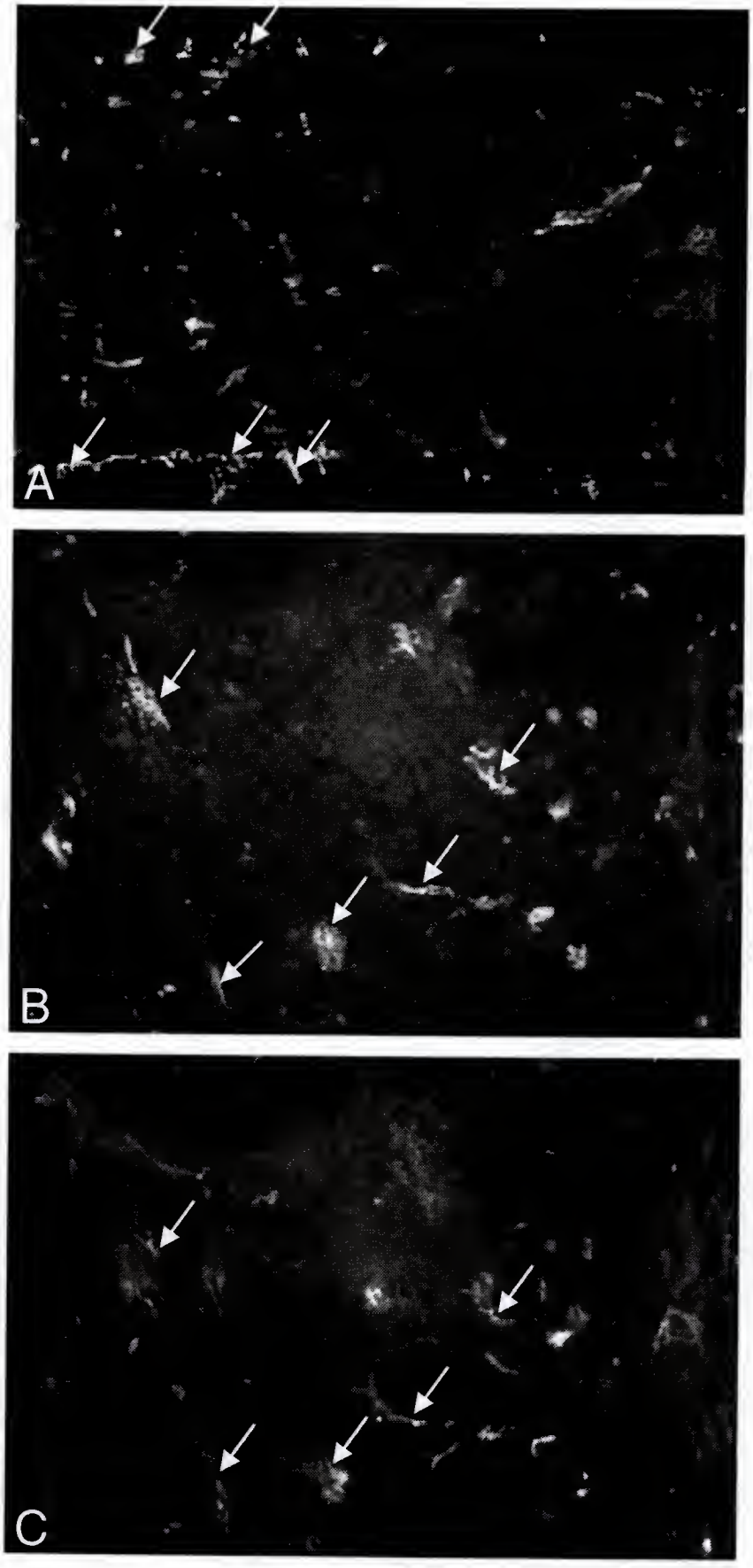
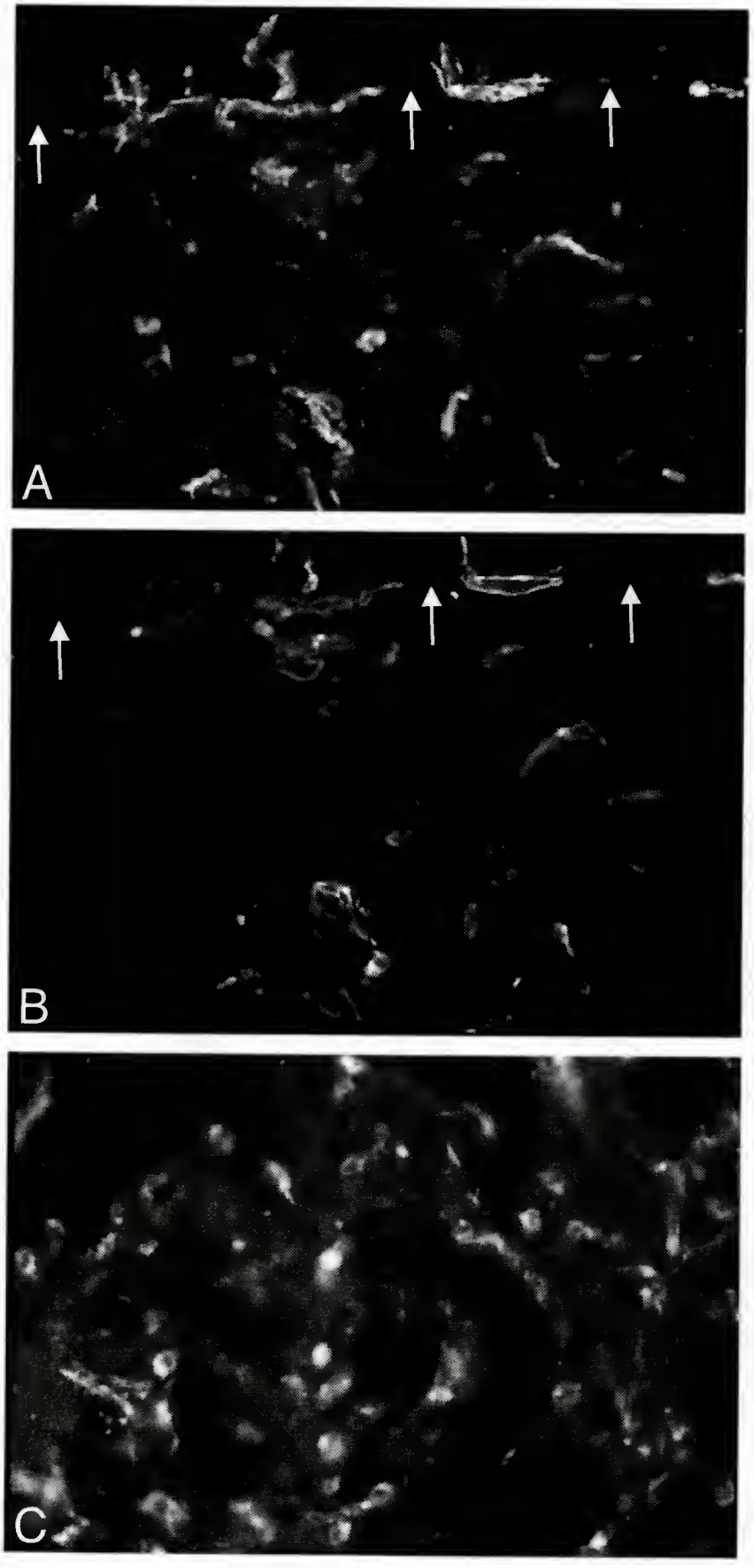

A
B
C

Fig. 3-15. High magnifications of astrocyte-impregnated PTFE implants. The tops of A,B show the lateral gelfoam-PTFE wall border (arrows) while the core of the matrix is shown lower. A,B represent the same fields of view seen though different fluorescent filters demonstrating remarkable coexistence of GFAP-IR elements (A) with laminin-IR profiles (B) resembling microvasculature (X120). Note that some of the GFAP-IR elements are seen penetrating the lacunae of the walls. Interestingly, these astrocyte implants also displayed faint OX-42-IR (C) in regions devoid of GFAP-IR cells (X120).


A
B
C

have significantly higher rankings than the microglial implants (Fig. 3-19). Nevertheless, both microglial and mixed glial implants had significantly higher neurofilament-IR (NF-IR) rankings (R and C) than did astroglial implants (Fig. 3-19). This is again illustrated in Fig. 3-20 which shows that this ingrowth was diametrically opposite to that seen in the control tubes.

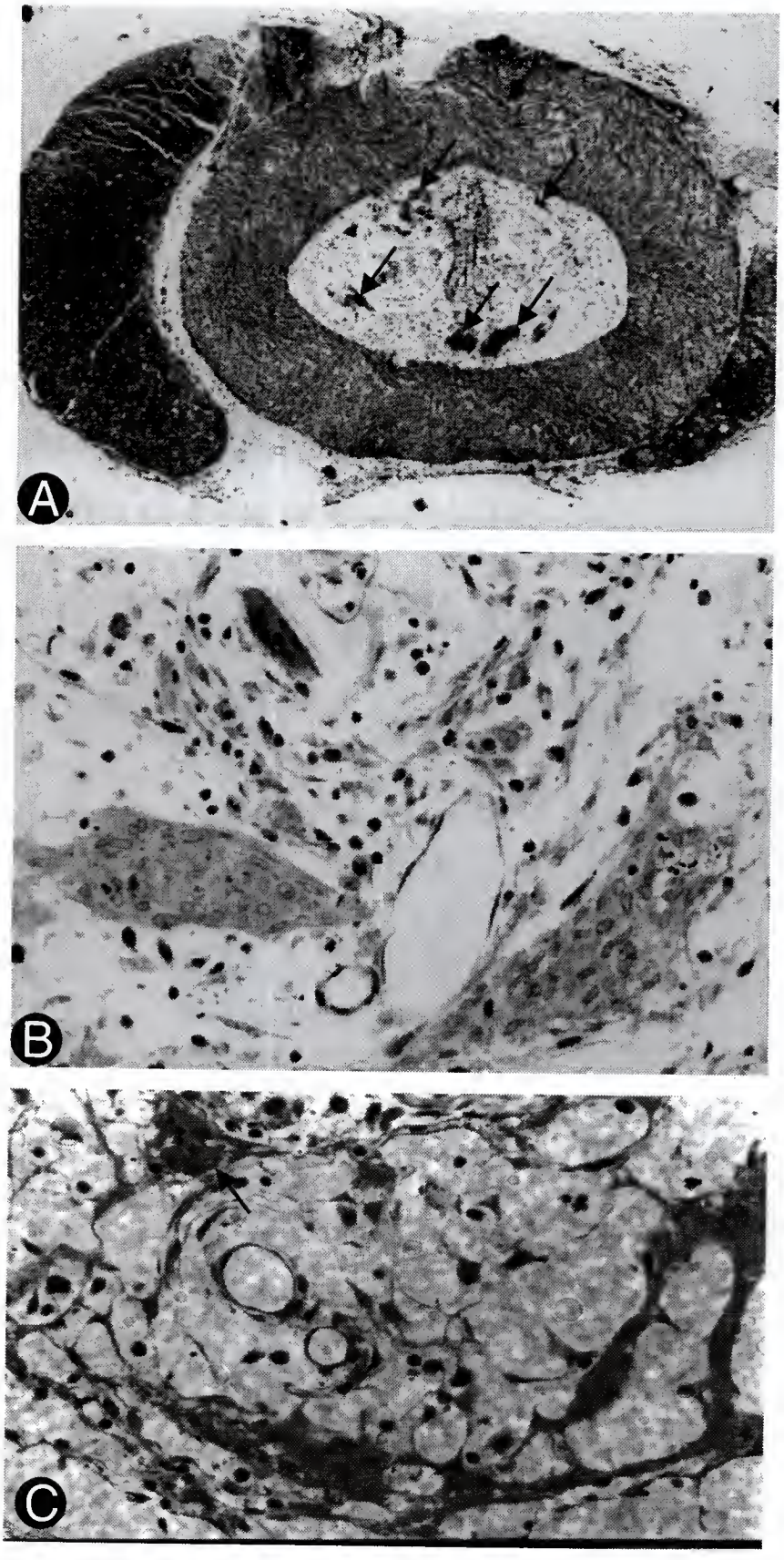
Neurotransmitter Characterization of Elongated Neurites in PTFE Tubes

Preliminary immunohistochemical investigations were performed to determine the neurotransmitter content of the ingrowing neurites which might give some clues as to their origin. The two antibodies chosen to characterize these processes were directed against calcitonin gene-related peptide (CGRP) recognizing ascending primary afferent fibers and 5-HT which labels descending serotonergic fibers.

Both stains demonstrated the normal distributions of these fibers within the host spinal cord surrounding the PTFE implants. In no instances were any 5-HT-IR processes seen penetrating into the gelfoam matrices or into the lacunae of the walls in control or experimental (i.e., cell-seeded) tubes. However, some were found to course along the edges of the tube outer walls.

On the contrary, CGRP-IR fibers penetrated both control and cell-seeded gelfoam matrices, but they only represented a relatively minor population of all the NF-IR processes seen in the implants, especially the grafts containing microglia. There did not appear to be any preferential ingrowth with repect to rostral or caudal ends, but qualitatively there seemed to be more CGRP-IR processes in the dorsal-most longitudinal sections.

Fig. 3-16. Transverse, plastic sections (2μm) through astrocyte-impregnated PTFE implants after 5 weeks. A. Low power photomicrograph shows regions in the graft containing large cell bodies (arrows) accompanied by various other cellular phenotypes (X10). B. Higher power reveals islands of cohesively packed astrocytes accompanied by macrophages and other elements in the center of these grafts (X320). C. All along the periphery of the gelfoam matrices was a trabecular meshwork of astrocytes that, in many instances, resembled an external glial limiting membrane. It was difficult to find neurites entering these grafts, and the few observed (arrow) were not found in astrocyte-rich regions (X320).


A
B
C

Discussion

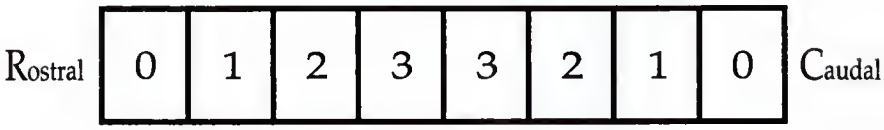
In the preceding investigation (Chapter 2), an initial attempt was made to determine whether activated microglia/brain macrophages derived from tissue culture (for simplicity herein referred to as microglia) would be permissive or nonpermissive to neuritic outgrowth in the injured spinal cord. The findings countered the view that reactive microglia in the injured CNS have neurotoxic effects since neuritic profiles were observed extending into microglia-seeded gelfoam matrices. Outgrowth also was ndicated in regions of inflammation, characterized by accumulations of OX-42-IR cells, seen in the host CNS immediately adjacent to biodegradable polymeric tubes housing some of the gelfoam matrices. The present findings are consistent with the previous observations regarding neuritic fiber penetration of cell-seeded matrices. Qualitatively and quantitatively, it was observed that gelfoam matrices seeded either with microglia alone or a combination of those cells and cultured astrocytes induced a greater degree of fiber ingrowth than seen either into control gelfoam implants or gelfoam containing enriched astroglial populations. Lastly, the LPS-activation of microglia *in vitro* prior to their transplantation failed to render these cells nonpermissive to axonal elongation.

PTFE vs. Biodegradable Polymeric Implants

One objective of this study was to overcome some of the complications introduced in the earlier investigation by the polymeric tubes that were used to introduce gelfoam matrices in a more oriented fashion. In particular, it appeared that a local inflammatory response had occurred as a consequence of polymer degradation. This may have caused an influx of endogenous microglia-derived brain macrophages and other cell types such that it was

Figure 3-17

SUMMARY OF ORDINAL DATA FOR EACH VARIABLE WITHIN IMPLANTATION GROUPS



IMPLANTATION GROUPS

VARIABLES		CONTROL		MICROGLIA		LPS-MICROGLIA		MIXED GLIA		ASTROCYTES	
		R	C	R	C	R	C	R	C	R	C
OX-42-IR	0	7	45	4	1	15	3	0	0	0	0
	1	40	65	31	38	1	24	0	0	0	0
	2	48	23	31	37	15	18	0	0	0	0
	3	62	24	115	105	79	68	70	70	54	54
		(N = 157)		(N = 181)		(N = 110)		(N = 70)		(N = 54)	
LAM-IR	0	18	41	3	4	9	0	0	0	3	0
	1	31	53	30	21	7	7	0	0	7	0
	2	54	29	40	71	13	20	0	0	12	0
	3	57	37	103	80	81	83	68	68	30	52
		(N = 160)		(N = 176)		(N = 110)		(N = 68)		(N = 52)	
NF-IR	0	41	69	16	24	18	2	1	0	23	0
	1	39	54	51	44	24	34	6	6	20	11
	2	44	23	38	52	25	46	12	12	6	12
	3	28	7	69	54	43	28	49	50	3	29
		(N = 152)		(N = 174)		(N = 110)		(N = 68)		(N = 52)	

Figure 3-18

QUARTILE VARIANCE (25% - 75%) OF ORDINAL DATA RANKING

	Control		Microglia		LPS-Microglia		Mixed Glia		Astrocytes	
	R	C	R	C	R	C	R	C	R	C
OX-42										
Rank	**3**	**1**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**
25th	1	0	3	3	3	3	3	3	3	3
M	2	1	3	3	3	3	3	3	3	3
75th	3	2	3	3	3	3	3	3	3	3
LAM										
Rank	**3**	**1**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**
25th	1	0	2	2	3	3	3	3	3	3
M	2	1	3	2	3	3	3	3	3	3
75th	3	2	3	3	3	3	3	3	3	3
NF										
Rank	**2**	**0**	**3**	**3**	**3**	**2**	**3**	**3**	**0**	**3**
25th	0	0	1	1	1	1	3	3	0	2
M	1	1	2	2	2	2	3	3	1	3
75th	2	1	3	3	3	3	3	3	1	3

Fig. 3-17,18. Representational summary of the immunohistochemical profiles that were observed within all the PTFE implants and given an ordinal ranking according to the procedure illustrated in Fig. 3-1. In brief, each implantation group had rostral (R) and caudal (C) sections of their tubes examined for each of the variable stains, and each tallied number in Fig. 3-17 represents individual sections given a rank (0-3; N=total sections counted). The shaded numbers are the ordinal rank most often ascribed to the variable immunostain and thus represents the rank assigned to each implantation group (R and C). These are highlighted in Fig. 3-18, which also displays the median (M) ranks along with the quartile variances. The assigned ranks for the implantation groups are schematically shown in Fig. 3-20.

Figure 3-19

"Mann-Whitney" U Test by Variable Group

I = Control II = Microglia III = LPS-Microglia IV = Astrocyte/Microglia V = Astrocytes

rOX-42

	I	II	III	IV	V
I		0.000137	0.000048	0.0000001	0.0000001
II			0.372514	0.000005	0.000036
III				0.001387	0.003213
IV					1.0000
V					

cOX-42

	I	II	III	IV	V
I		0.0000001	0.0000001	0.0000001	0.0000001
II			0.709988	0.000002	0.000028
III				0.000079	0.000386
IV					0.966649
V					

rLAM

	I	II	III	IV	V
I		0.000069	0.000001	0.0000001	0.019245
II			0.075368	0.000001	0.757298
III				0.003027	0.133728
IV					0.000071
V					

cLAM

	I	II	III	IV	V
I		0.0000001	0.0000001	0.0000001	0.0000001
II			0.000018	0.0000001	0.0000001
III				0.005771	0.011792
IV					1.000000
V					

rNF

	I	II	III	IV	V
I		0.000113	0.010890	0.0000001	0.000050
II			0.565099	0.000006	0.0000001
III				0.000021	0.000001
IV					0.000001
V					

cNF

	I	II	III	IV	V
I		0.0000001	0.0000001	0.0000001	0.0000001
II			0.524300	0.0000001	0.002448
III				0.0000001	0.007706
IV					0.034986
V					

Figure 3-20

SCHEMATIC SUMMARY OF IMMUNOHISTOCHEMICAL PROFILES WITHIN PTFE IMPLANTS USING ORDINAL DATA

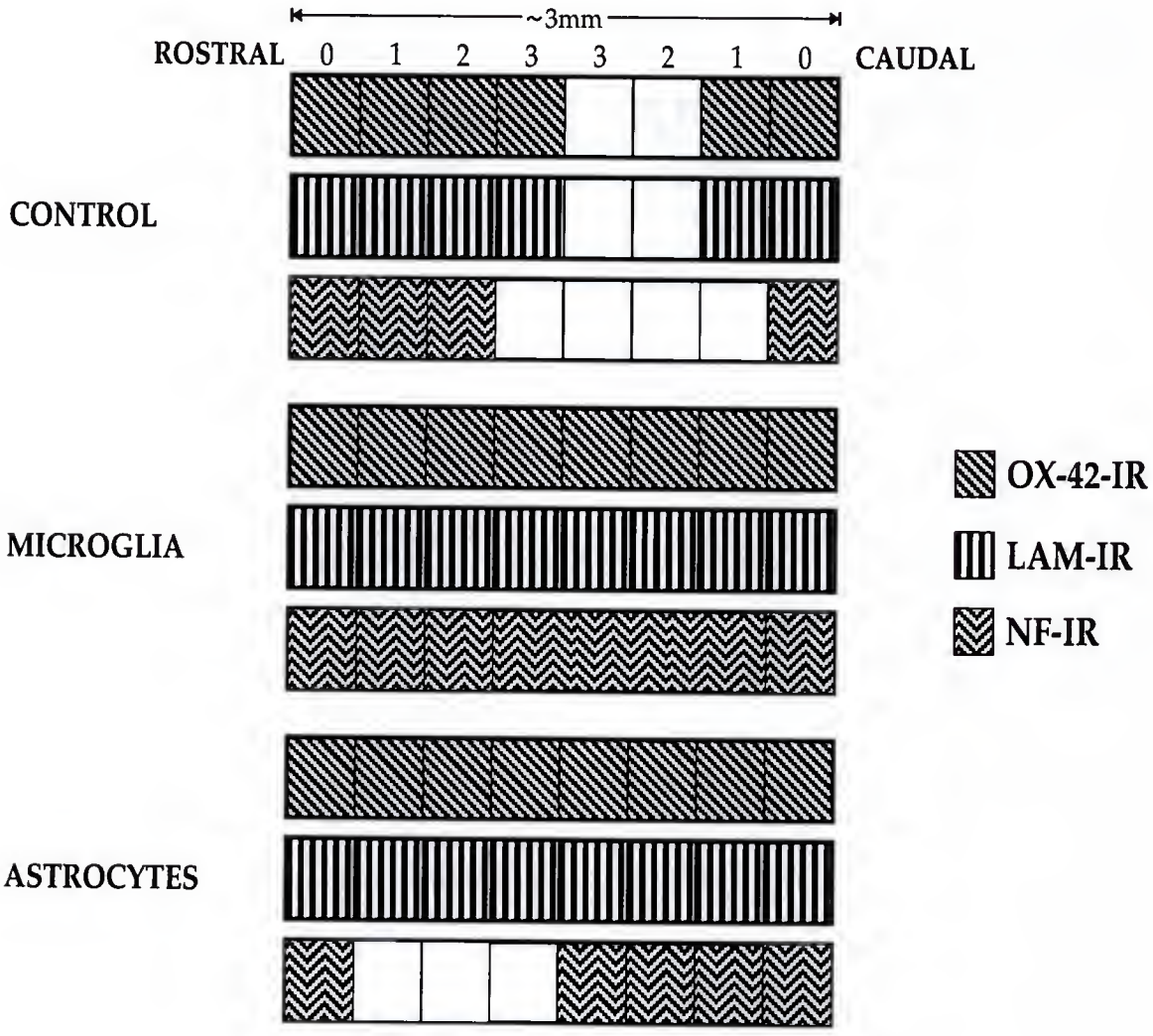


Fig. 3-19,20. A "Mann-Whitney" U test was used to determine the significant difference (P values) between each implantation group for all three variables, rostrally and caudally (i.e. r0X-42, cOX-42, rLAM, cLAM, rNF, cNF). The P values are shown in Fig. 3-19. Note that the microglial implantation groups had signifiacanly higher rankings in all R and C variable stains than the control implants. This is illustrated in Fig. 3-20. However, there were no significant differences between the LPS-stimulated versus non-stimulated microglial implants, except in cLAM. In addition, both microglial and mixed glial implants had significantly higher rNR and cNF rankings than astroglial implants, and the neuritic ingrowth into control tubes was diametrically opposite to that seen in astrocytic tubes, again illustrated in Fig. 3-20.

unclear to what cell or tissue type(s) the observed neuritic ingrowth could be attributed. Conceivably, breakdown of the polymer also could have promoted enhanced activation of both exogenous and endogenous microglia. Thus, it could not be judged whether neuritic ingrowth was due to augmented microglial/brain macrophage properties or conversely whether fiber elongation was actually being minimized and, accordingly, less robust than it may have been without additional microglial stimulation.

The PTFE tubes used in the present study did not appear to induce a local cellular response. This is consistent with previous observations derived from the use of Gore-tex tubes in studies of peripheral nerve regeneration (Valentini et al., 1989; Young et al., 1984). Although a direct quantitative comparison cannot be made between these and previous results (Chapter 2), the stability of the PTFE polymer did not appear to contribute to any qualitatively greater degree of neuritic ingrowth into either control or experimental gelfoam matrices than seen in the biodegradable tubes (Chapter 2). The potential effects of additional microglial stimulation, noted above, also seem unlikely given that grafts of LPS-activated microglia did not alter neuritic ingrowth relative to the effects observed with unstimulated cells.

Cellular Contributions to Neuritic Ingrowth

On the other hand, PTFE tubes did not eliminate the possibility of other cell types playing a role in the neuritic growth response observed. While prelabelling of microglia successfully demonstrated that many of the grafted cells were retained in the implants, Hoechst 33342 labeling showed that cells, presumed to be largely host-derived, had entered the PTFE tubes via the porous channels of the tube walls. In addition, host cell infiltration of the tubes occurred at the rostral and caudal open ends. That cells within the injured spinal cord could promote some degree of ingrowth was indicated, as

before (Chapter 2), by NF-IR profiles in control matrices. That mature astrocytes may be involved seems unlikely since no GFAP-IR elements were seen in the implants. Furthermore, as will be discussed in more detail below, implants of enriched cultured astroglia had no significant neurite growth-promoting effects. A contribution of oligodendrocytes can be readily eliminated in light of the substantial evidence that these cells and the myelin sheaths they produce can be nonpermissive to axonal elongation in the CNS. In addition, histological inspection of semithin plastic sections failed to provide any indication of CNS myelin profiles around axons inside these tubes. While the possible involvement of ependymal cells, known to show some responses to spinal cord injury (Matthews and Gelderd, 1979; Guth et al., 1985), cannot be totally excluded, none of the observations obtained thus far offer any support to this consideration.

Since OX-42-IR cells had robustly invaded control gelfoam implants and because microglia-seeded matrices promoted an even greater response (Fig. 3-9), it would appear that microglia-derived brain macrophages are playing a significant neurite growth-promoting role. However, whether they are directly involved in fiber elongation is uncertain, and a more systematic longitudinal study of axonal-cellular interactions at early post-implantation intervals could help resolve this issue.

As previously stated (Chapter 2, Discussion), it is conceivable that microglia/brain macrophages may be instrumental in mobilizing other cell types with demonstrated inherent growth-promoting properties. In that regard, the most likely candidates are Schwann cells, connective tissue elements and vascular constituents. Of these, the most tempting candidate is the former because of: (i) the established ability of these cells to facilitate axonal regeneration in the PNS (Richardson et al., 1980; David and Aguayo,

1981; Aguayo et al., 1982) and CNS (Kromer and Cornbrooks, 1985; Paino and Bunge, 1991; Paino et al., 1994; Xu et al., 1995), (ii) the capacity of these cells to produce laminin which was observed in the gelfoam matrices, and (iii) histological observations from 2 μm plastic sections showing myelinated axons with surrounding Schwann cell cytoplasm. Schwann cells could have gained access as a result of perivascular nerve damage or to ventral or dorsal root injury consequent to the spinal lesions. The presence of laminin-IR in the implants, with which some NF-IR profiles seemed to be in close register, also may be associated with neovascularization in which case BL-producing endothelial could be involved. On the other hand, microglia-derived brain macrophages may have contributed to the formation of the loose connective tissue matrix seen in plastic 2 μm sections. This could have provided an essential framework for other cellular infiltrations that in turn could have provided a stimulus for neuritic ingrowth. Unfortunately, no evidence presnetly exists to suggest which single or multiple cell types are involved in the actual neuritic growth process.

While, the presence of laminin-IR may provide clues, the pattern of this ECM molecule's deposition in the matrices was unusual even though definite subpial perivascular and external limiting membrane staining was seen in the host cord. That OX-42-IR cells may be producing laminin was noted, but this was more the exception than the rule. However, the production of TGF-β1 by activated microglia in culture and in the injured CNS is thought to enhance cell migration, cell proliferation, and the production of ECM components (Roberts & Sporn, 1990), similar to its role in peripheral wound healing (Mustoe et al., 1987). In fact, TGF-β has been shown to act as a mitogen for Schwann cells (Ridley et al., 1989), and in a microglia-

enriched environment devoid of GFAP-IR cells these peripheral elements might invade and develop interactions with ingrowing neurites.

Thus, at best, it can only be suggested at the present time that neuritic ingrowth appears to entail some relationship between OX-42-IR microglia/brain macrophages and the deposition of laminin since qualitatively and quantitatively the staining patterns of these elements were in register with the extent of axonal ingrowth in both control and various microglia-seeded matrices. Again, this speaks to the need for a more orchestrated time-course study in which the earliest neuritic ingrowth can be defined and characterized relative to the immediate cellular surround.

Effects of Grafted Astrocytes

The rapid induction of laminin following CNS injury suggests that adult astrocytes have the ability to produce this glycoprotein (Liesi et al., 1984; Liesi and Risteli, 1989), the role of which in the lesioned CNS has not been identified. These authors suggest that the induction of laminin in astrocytes might be involved in scar formation or that laminin might be specifically needed during the early phases of CNS tissue repair. Strong expression of laminin and tenascin has been reported by others in reactive astrocytes of the traumatized rat spinal cord, and axons in the glial scar showed an intimate relationship to astrocyte-associated laminin, as well as to tenascin-reactive glia (Risling et al., 1993). Neurites predominantly followed processes showing laminin-like immunoreactivity, suggesting that scar tissue may support and guide regrowing axons after spinal cord injury. The neuritic ingrowth seen in this study also was associated with laminin-IR profiles in many instances but never with GFAP-IR cells in microglial grafts.

While a varying degree of axonal ingrowth was observed in virtually every experimental and control implant, it was of interest that neuritic

elongation into a gelfoam matrix was most challenged when seeded with enriched populations of cultured astrocytes. This finding is in fundamental agreement with the view that astroglia may exert inhibitory or nonpermissive influences affecting neuritic elongation in the injured CNS (Reier et al., 1983; 1988; Liuzzi and Lasek, 1987). On the other hand, the results are at odds with other observations showing that astroglial monolayers in tissue culture can support neuritic elongation (Fallon, 1985), as well as some *in vivo* findings indicating a growth-promoting effect of reactive astrocytes in the injured hippocampus (Fagan and Gage, 1990). It is not unlikely, however, that once astroglia are placed into an intraspinal lesion environment they may either lose neurite-supporting properties or assume characteristics that are adverse to axonal elongation. It certainly was apparent that the expression of GFAP by these cells was influenced by the local microenvironment. The expression of GFAP in astrocytes varies depending on developmental stage, culturing conditions, and whether the CNS has been traumatized in any way (see Eng et al., 1987). Therefore, it is conceivable that other proteins expressed by astrocytes can be modulated by the surrounding cellular milieu.

The fact that axonal elongation had nevertheless occurred in some regions of astroglial-seeded tubes casts a different light on this consideration. Infiltrating host microglia/brain macrophages, however, could have partially modulated the astrocytic effect. This interpretation is supported by the results of transplanting a combination of microglia and astrocytes that were co-seeded in gelfoam; NF-IR profiles appeared in these matrices to an even greater extent than seen with other microglial-seeded grafts.

A perplexing observation derived from the astroglial implants was that a differential degree of NF-IR profile ingrowth was found at opposite tube ends. As seen in Fig. 3-20, more neuritic penetration was observed caudally

than rostrally. There is no immediate definitive explanation for this result, but it is of interest that a greater degree of caudal NF-IR was observed in a recently published report (Wang et al., 1995) describing the effects of astroglial implants in the injured rat spinal cord. It was proposed that this might reflect greater regenerative tendencies of ascending dorsal root fibers, but this is questionable since descending sensory collaterals should have similar growth capacities. These authors also noted a high degree of caudal migration of astroglia that were originally seeded and grafted to the spinal cord in a gelfoam pledget. In that event, their results would suggest that transplanted astroglia from tissue culture can be conducive with regeneration by modifying the host tissue in some way. To put this seemingly disparate interpretation in agreement with the findings of the present study, a similar caudal migration may have occurred with the mobilized astroglia being modified in their neurite growth-promoting properties by interactions with infiltrating microglia/brain macrophages.

Extent, Long-Term Persistence, and Origin (s) of Ingrowing Neurites

While an association between microglia/brain macrophages and neuritic elongation has been suggested by these and previous results (see Chapter 2), the robustness of this neuronal response is still uncertain. At the early time points of the present study many axons extending into the implants may be unmyelinated in which case labor-intensive axonal counts at the ultrastructural level would be necessary to appreciate fully the extent of the proposed neurite growth-promoting effect. Using Schwann cell or peripheral nerve implants as a gold standard (Aguayo et al., 1982; Paino et al., 1994; Xu et al., 1995), the impression is that at 5 weeks, the extent of axonal ingrowth is far less with microglial-associated implants. This difference, however, may be related to some property of gelfoam in which case it would

be worth repeating these experiments with other matrices (e.g., Matrigel) as used for the introduction of enriched Schwann cell populations. Alternatively, examination of NF-IR into implants maintained over longer post-implantation intervals may be as instructive. Analysis of a more protracted time-course also would demonstrate to what degree these fibers persist. While they may die-back eventually because of failure to reach an appropriate target, the same result could occur if ultimately microglia began to express more toxic-like effects.

Pending the outcome of such investigations, the sources of neurites growing into these tubes becomes of greater interest as does the possibility of engineering growth through microglia-seeded tubes placed into the site of a complete spinal cord transection. The sources of the neurites seen in the implants have not yet been completely characterized, but a small percentage have been shown to be CGRP-IR. This correlates with observations in plastic sections that some fibers seemed to originate from dorsal roots. No serotonergic fibers (5-HT-IR) were found in any of the implants though many were found coursing along the outside edges of the PTFE tube walls.

Injured, adult rat motor neurons have been shown to regenerate through white matter into nearby ventral rootlets after axotomy within the substance of the spinal cord (Risling et al., 1983). Based on their influence on regenerating motoneurons (Streit et al., 1988; Streit, 1993), the microglial implants may have provided a conduit for axotomized motor axons to grow through. Cholinergic staining may establish whether some of the ingrowing neurites are motor axons or of other intraspinal origins. However, the potential exists that the neurofilament staining recognized dendritic processes or (para)sympathetic fibers associated with penetrating microvascular elements, especially in light of the observed colocalization of

penetrating neurofilament-IR processes and laminin-IR elements. Staining with an antibody against vasointestinal polypeptide (VIP) or substance P (SP) may provide insight into the origins.

CHAPTER 4
TRANSPLANTATION OF FLUORESCENTLY-LABELED MICROGLIA INTO THE ADULT RAT SPINAL CORD

Introduction

The influence that Schwann cells, astrocytes and oligodendrocytes exert on the guidance, elongation and regeneration of central nervous system (CNS) axons has been investigated using various transplantation and injury paradigms (Bernstein and Goldberg, 1989; Crang and Blakemore, 1991; Franklin et al., 1991; Martin et al., 1991; Paino and Bunge, 1991; Smith and Silver, 1988; Wang et al., 1995; Xu et al., 1995). Interestingly, despite the abundance and rapid accumulation of microglia/brain macrophages at sites of CNS injury and the numerous protocols for their isolation in culture, transplantation studies of this population of CNS glia have not been pursued. Microglia have been shown to secrete diverse cytokines/growth factors, some of which have neurite-growth promoting activity (Lindholm et al., 1992; Mallat et al., 1989; Shimojo et al., 1989). In light of *in vitro* studies that suggest microglia produce neurotoxic molecules (Banati et al., 1993), the logical method to resolve this disparity is to transplant this cell population into the injured CNS.

In the two preceding chapters (Chapters 2 and 3), it was found that grafts of microglia/brain macrophages, derived from tissue culture, did not exert an overtly negative influence on neuritic elongation in the injured spinal cord. One disadvantage, however, of transplantation experiments

involving cells with counterparts in the host CNS is the inability to differentiate host from donor elements. Consequently, the nature of cellular interactions, extent of donor cell survival, and migratory behavior of the implanted elements cannot be easily determined. Therefore, similar to experiments done with other glial cells (Goldberg and Bernstein, 1988; Wang et al., 1995; Xu et al., 1995), suspensions grafts of enriched microglia labeled with fluorescent dyes were injected into adult rat spinal cords in order to examine their short-term survival and possible migration. The results of this study have been previously summarized (Rabchevsky et al., 1993).

Methods and Materials

Isolation of Microglia

Microglia were isolated for tissue cultures using methods previously described in Chapter 2. Briefly, this entails the culturing of dissociated neonatal rat brains in DMEM-10% FBS for several weeks by which time the cultures are primarily characterized by mixed glial populations of microglia and oligodendrocytes lying upon a bed of confluent astrocytes. Microglia tend to float off of the underlying astrocytic layer more than oligodendrocytes, so they can be isolated from the supernatants every 3 days and enriched to >95% purity after selective plating.

Pre-Graft Labeling of Microglial Suspensions

Pellets of enriched microglia were resuspended in 2 ml of DMEM and incubated with either 0.04% Fluorogold (FG) or 5 μg/ml of DiI-labeled acetoacetylated LDL (DiI-ac-LDL; Biomedical Technologies, Inc.) for 1-2 hrs. at 37^{0}C while gently agitating. The suspension was then diluted, pelleted and washed twice with DMEM before adjusting to ~50,000 cells/μl for injections

and culturing of small samples for further immunocytochemical characterization. Viability of the cells before transplantation was assessed with Trypan Blue exclusion to be >95%. Cultures of the uninjected suspensions verified that >90% of the grafted cells were positive for both GSI-B4 isolectin and DiI-ac-LDL.

Surgeries and Injections

Adult female Sprague-Dawley rats (N=9) were anesthetized with Ketamine (90 mg/kg, i.p.) and Xylazine (10 mg/kg, i.p.) and a laminectomy was performed at the 10th vertebral level. A small incision was then made through the dura to expose the spinal cord surface. A 33 gauge Hamilton syringe was filled with the prepared microglial suspension and then stereotactically inserted into the normal spinal cord 0.5 mm lateral to midline and 1-2 mm deep into the spinal cord. Over the course of 3-5 minutes, 3 µl of suspension was injected and the needle was left undisturbed for an additional 2 minutes to minimize reflux. The syringe was then carefully withdrawn and the animals' muscle and skin were sutured. After survival times of 1, 3, 4 or 7 days the animals were perfused with PBS containing heparin and sodium nitrate, followed by 4% paraformaldehyde in PBS.

Animal Categorization

Some rats were injected with microglia prelabeled with either FG or DiI-ac-LDL and sacrificed after 1 (N=2) and 3 days (N=2). Others were injected with DiI-ac-LDL-labeled microglia and sacrificed after 4 (N=1) and 7 days (N=2). Control injections consisted of microwave-killed DiI-ac-LDL-labeled microglia (N=1) and DMEM containing 5 µg/ml DiI-ac-LDL (N=1).

Histochemistry

In Vitro. Glial cultures were maintained either on glass coverslips or 35 mm plastic petri dishes. Before staining, the cultures were washed twice

with PBS and then fixed with 4% paraformaldehyde/PBS (PF) for 30 min. The plates were then washed with PBS and incubated with mouse anti-GFAP (1:400; Sigma) and/or biotinylated GSI-B4 isolectin (10 μg/ml, Sigma) in PBS for 1 hr. at room temperature. The plates were again washed several times with PBS and then incubated with either donkey anti-mouse-TRITC (1:300, Jackson) or biotinylated anti-mouse-IgG (1:200, Vector) for 1 hr. Then, if needed, strept-avidin-FITC (1:200, Vector) for another 60 min. After final washes, cultures were mounted with glycerin/PBS.

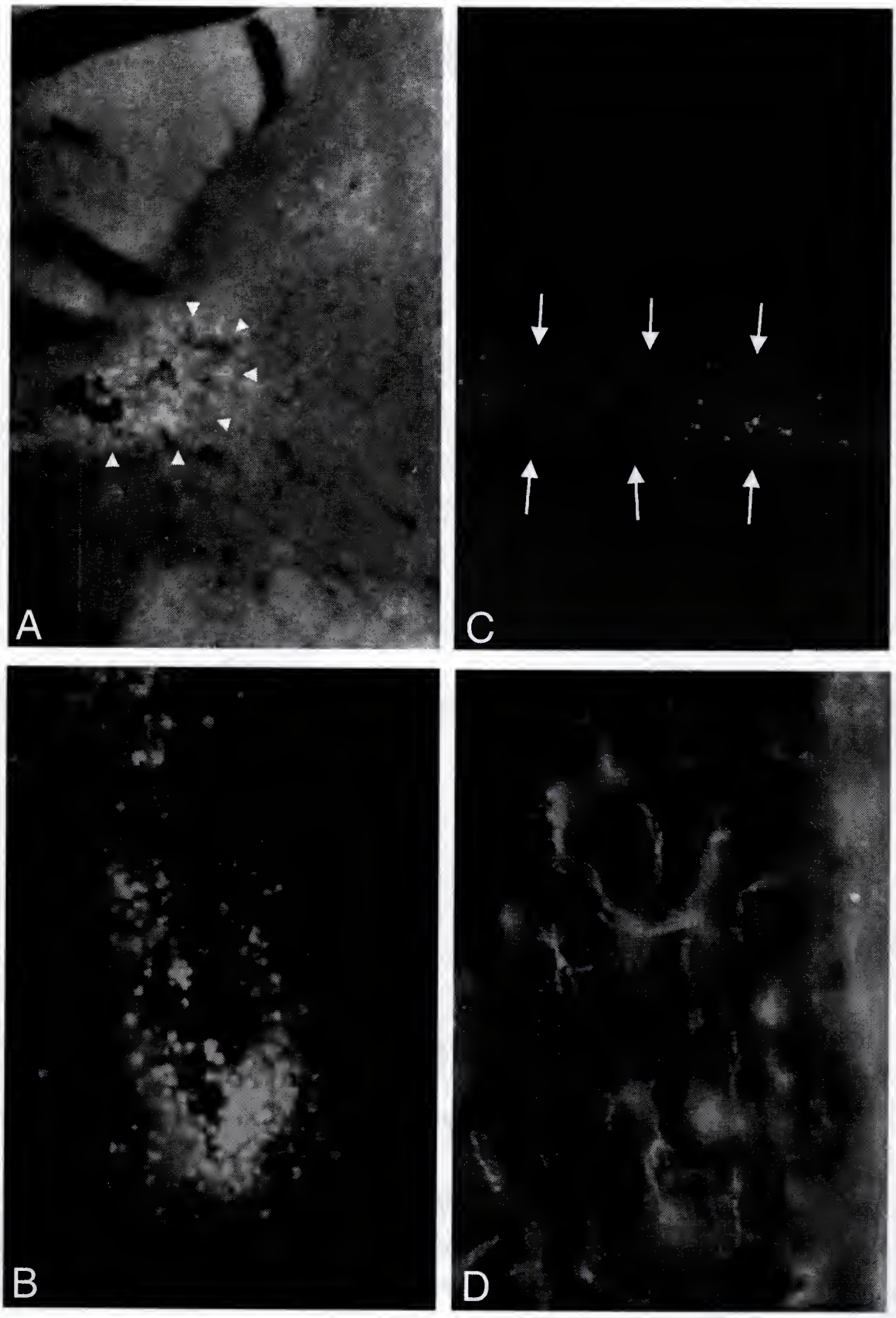
In Situ. Spinal cords were post-fixed in 4% PF for several days before cutting 20-40 μm sections on a vibratome and incubating the freshly cut sections in micro-wells containing mouse anti-GFAP and/or GSI-B4-FITC (Sigma, 10 μg/ml PBS) overnight at 4°C. The sections were then washed and either mounted onto gelatin-coated slides or were incubated with anti-mouse antibodies (see above) for an additional 2 hrs. at room temperature. After final washes and mounting, the slides were allowed to air dry. They were then briefly rehydrated with PBS and coverslipped with glycerin/PBS. Stained cultures and vibratome sections were viewed under a Zeiss microscope equipped with epifluorescent illumination (Axiophot filters: #01-UV; #09-FITC; #14-RITC).

Results

Characterization of Microglial Transplants

Syngeneic rat spinal cords were injected with cultured microglial cells that were prelabeled with either DiI-ac-LDL or Fluorogold. Each injection proved successful upon immunofluorescent, microscopic examination. The first trial included four animals injected with microglia that were prelabeled

Fig. 4-1. Assessment of microglial graft survival. A. In a cross section of a spinal cord (dorsal surface is at the left), 3 days after the injection of labeled microglia, there is a distinct epicenter of GSI-B4-FITC-IR cells seen (arrowheads delineate graft; X40). B. Higher magnification of same injection site (dorsal surface is at the top), but viewed through a different band pass filter to reveal DiI-ac-LDL-labeled microglia (X200). Figures C and D show horizontal sections of a spinal cord injected with labeled microglia 4 days prior to perfusion fixation. C. Note the scattered pattern (arrows) of labeled cells along the rostro-caudal axis in the ipsilateral dorsal columns (X20). D. Higher magnification of the labeled cells reveals that they are GSI-B4-FITC-IR microglia (X320).


A
C
B
D

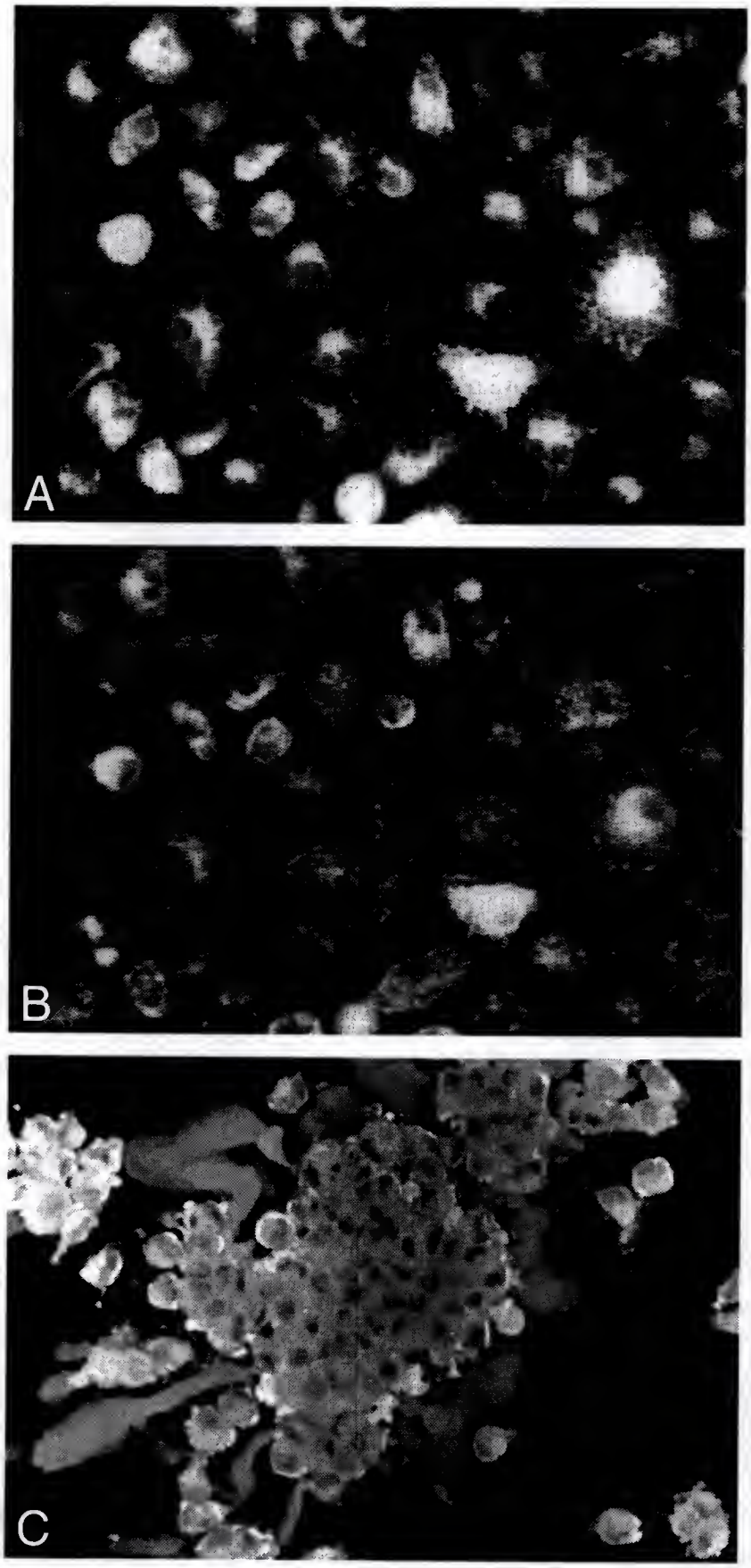
with one of the dyes. After 1 or 3 days, the injection sites were characterized by clusters of fluorescently-labeled cells with prominent nuclei that stained with the GSI-B_4-FITC lectin (Fig. 4-1A). Lectin staining also revealed morphological activation of endogenous microglia around the injection site and in the contralateral cord after 3 days. The DiI-ac-LDL-labeled microglia fluoresced far more intensely than did the FG-labeled cells, and this was most evident with the 3-day injections (Fig. 4-1B). Leeching of both the dyes within the spinal cord sections was evident but more prominent with the FG prelabel as evidenced by its labeling of motoneurons and other host cells not seen with the DiI-ac-LDL.

Three more spinal cords were then injected with DiI-ac-LDL-labeled microglia and sacrificed after 4 or 7 days. Longitudinal, horizontal sections made through the 4 day spinal cord and one of the 7 day cords revealed the apparent migration of transplanted microglia into white matter (Fig. 4-1C). The other 7 day animal had coronal sections made through the injection site that suggested the lateral migration of DiI-ac-LDL-labeled microglia into white matter away from the injection epicenter that was still populated by clusters of ameboid microglia. The ramified forms of DiI-ac-LDL-labeled microglia found at more distant locations from the injection site, mostly in the dorsal funiculi, were GSI-B4-FITC-IR (Fig. 4-1D).

Control Experiments

The remaining uninjected cell suspensions were plated onto 35 mm petri dishes. After 7 days *in vitro* (DIV) the cells were still robustly labeled with the DiI-ac-LDL (Figs. 4-2A,B). However, the physical displacement of labeled cells in culture (Fig. 4-2C) and co-culturing experiments of DiI-ac-LDL-labeled microglia with non-labeled microglia revealed that the receptor-mediated uptake of DiI-ac-LDL was unstable and the dye could be

Fig. 4-2. Characterization of DiI-ac-LDL-labeled microglia. A,B. Culture of microglia plated immediately after surgery, and then fixed 3 days later for lectin staining. Both are the same field, but viewed through different band pass filters to reveal intracellular DiI-ac-LDL in A, and the extracellular GSI-B4-FITC stain in B (X320). C. A coverslip placed on top of a cluster of DiI-ac-LDL-labeled microglia demonstrates the physical leeching of the dye into the culture medium (X320).


A
B
C

accumulated by non-labeled microglia after 3 DIV. These results suggested that the pressure injection method employed most likely produced a similar effect in the confines of the normal spinal cord. Therefore, subsequent *in vivo* control injections of either heat-killed, DiI-ac-LDL-labeled microglia (N=1) or DiI-ac-LDL in vehicle (N=1) were performed to assess the stability of the dye *in situ*. These control injections also showed leeching of the intracellular dye as evidenced by labeling of endogenous populations of both ameboid and ramified microglia around the epicenter of the control vehicle-DiI-ac-LDL injection However, it was interesting to find that unlike the ramified forms of DiI-ac-LDL-labeled microglia present in distal white matter of experimental cords, the control, microwave-killed cell injection labeling was primarily limited to ameboid cells confined to the parenchyma surrounding the injection sites and to necrotic cells lacking prominent nuclei within the epicenter.

Discussion

These experiments have established the first attempts to transplant suspension grafts of enriched microglia into the adult rat CNS. Modified protocols were developed for the isolation of microglia from mixed glial cultures and immunocytochemistry was used to characterize individual cellular elements comprising the suspension grafts. The results suggest that grafted microglial cells survived in the rat spinal cord for at least one week, and that Fluorogold was an ineffective marker for transplanted microglia due to diffusion and non-specific labeling *in vivo*. DiI-ac-LDL proved to be a more specific and robust intracellular label for transplanted microglia, and initial observations indicated their survival, migration and differentiation.

This was evidenced by the appearance of DiI-ac-LDL-labeled ramified microglia particularly along distal white matter tracts 1-2 mm rostral and caudal of the injection site after 4 days.

Co-culturing experiments of DiI-ac-LDL-labeled microglia with non-labeled cells revealed that the receptor-mediated uptake of DiI-ac-LDL was fragile since the dye was released into the culture media and taken up by non-labeled microglia. The vehicle (DiI-ac-LDL) control injection also suggested leeching of the dye as seen by the labeling of endogenous ameboid and ramified microglia around the injection site. This differs with other findings suggesting that only ameboid microglia have LDL receptors (e.g. Giulian et al., 1989), and perhaps indicates that endogenous "resting" microglia become activated after transplantation and begin to express LDL receptors (Giulian, 1987). Interestingly, the, microwave-killed injections predominantly labeled the necrotic epicenter and ameboid cells around the injection, and few labeled cells were found distant from the injection site.

While it is tempting to suggest that the labeled cells found in the distal dorsal columns and lateral white matter had migrated from the transplant, the *in vitro* and *in vivo* control experiments showed that the distinction between host and DiI-ac-LDL-labeled grafted microglia must be approached with caution due to possible leeching of the dye into the host. It that sense it should be noted that some of the ameboid microglia in the epicenter of the injection sites may have been host-derived brain macrophages that invaded the region and took up the dye exuded by grafted cells. On the other hand, it is difficult to imagine that none of the labeled cells were graft-derived or that the dye would predominantly label endogenous microglia, especially after the one day injections. In no instance were cells immunoreactive (IR) for glial

fibrillary acidic protein (GFAP) ever labeled with DiI-ac-LDL, and every injection site was surrounded by reactive astrocytes.

In conclusion, although these experiments suggest the feasibility of transplanting cultured microglia into the rat spinal cord, in order to truly differentiate grafted microglia from host cells it will be necessary to incorporate a reporter gene, such as β-galactocidase (β-gal) or green fluorescent protein (GFP), into the grafted microglia isolated from cultures. This seems a feasible approach in light of the proliferative capabilities that microglia possess, especially in culture when they are exposed to factors such as IL-3, colony stimulating factors (CSF-1, GM-CSF), or conditioned media containing cytokines. This is an approach that will optimize all future transplant studies that attempt to prelabel and differentiate graft cells from analogous host elements.

CHAPTER 5
OVERVIEW

The implantation paradigms developed in this thesis showed that cultured microglia transplanted into the injured rat spinal cord created a microenvironment that promoted neuritic growth, vascularization, and infiltration of numerous host cellular elements and laminin 2-5 weeks after implantation. More importantly, they demonstrated that microglia are neither neurotoxic nor do they inhibit neuritic outgrowth in the injured spinal cord. Control implants which contained cell-free Gelfoam matrices demonstrated scarce neurofilament- and laminin-IR elements, and only in register with the infiltration of host-derived OX-42-IR cells. In addition, astroglial implants showed less neurite growth-promoting actions than either microglial or mixed glial grafts, suggesting that microglia can modify the putative inhibitory effects that astrocytes may have on growing axons during gliosis.

The hypotheses regarding the observed neuritic growth and cellular infiltration into gelfoam matrices impregnated with microglia or microglia mixed with astrocytes are related to the activation states of microglia, their known production of cytokines/growth factors, or the elaboration of laminin and other ECM molecules. For example, the production of TGF-β1 by activated microglia in culture and in the injured CNS is thought to enhance cell migration, cell proliferation, and the production of ECM components (Roberts & Sporn, 1990), similar to its role in peripheral wound healing (Mustoe et al., 1987). In fact, TGF-β has been shown to act as a mitogen for

Schwann cells (Ridley et al., 1989), and in a microglia-enriched environment, devoid of GFAP-IR cells (astrocytes), they might invade and develop interactions with ingrowing neurites. Although the invasion of Schwann cells into the spinal cord is a nonspecific phenomenon, infiltrating Schwann cells most likely originate from perivascular spaces in the spinal cord, spinal roots damaged during implantation or multipotential mesenchymal elements of the CNS (Kao et al., 1983).

Two relatively modest regenerative processes follow severance of the spinal cord. Terminal sprouts are formed by cut axons and, when the transection is incomplete, collateral sprouts may come from adjacent undamaged axons (Puchala and Windle, 1977). Passage of axonal sprouts through damaged tissue of the spinal cord depends on the presence of appropriate elements to provide guidance. Changes in the balance of positive versus inhibitory factors may alter the local environment and, ultimately, influence the amount of regeneration that takes place in the CNS following injury (Smith et al., 1986). Strategies should be developed to enhance the growth-promoting components of gliosis while limiting the expression of inhibitory molecules.

Successful regeneration of severed nerves in the invertebrate CNS is accompanied by increased laminin-IR that is coincidental with the appearance of numerous microglial cells both in time and distribution (Masuda-Nakagawa and Wiedemann, 1992; Masuda-Nakagawa et al., 1993a,b). Laminin is a large complex of three very long polypeptide chains arranged in the shape of a cross and held together by disulfide bonds. It consists of a number of functional domains: one binds type IV collagen, one to heparan sulfate, and one or more to laminin receptor proteins on the surface of cells. Laminin supports neurite outgrowth from normal dissociated CNS neurons,

while it supports nerve fiber outgrowth from dissociated PNS neurons only when combined with fibronectin (Rogers et al., 1983). Whether microglia actively played a role in the deposition of laminin seen in the experiments of this thesis or whether the microglia created an environment for invasion of ECM-secreting cells, such as Schwann cells that secrete BL, is unclear. However, the contention that the BL covering astrocytes within scar tissue may reduce axon-astrocyte interactions thought to be essential for the guidance of neurites (Smith et al., 1986) is not supported by the coexistence of laminin-IR elements and neurites within microglial grafts devoid of any GFAP-IR cells.

The microglia-impregnated implants that were stimulated with LPS induced similar vascularization and neuritic growth into their matrices compared to unstimulated implants. The lack of remarkable differences may be due to the down regulation of the activated cells after being introduced into the lesioned spinal cord. It also may be that the amount of stimulated cells within the these implants were vastly out numbered by activated host microglia/brain macrophages attracted into the implants that secrete their own injury-induced cytokine/growth factors.

Several decades ago, it was discovered that the density of the astrocytic scar was reduced, and neuritic regeneration was enhanced after injection of a pyrogenic bacterial polysaccharide (LPS) into spinal cats (Windle, 1956). Injection of such an agent into the CNS results in the morphological activation of resident microglia (Andersson et al., 1992), which are thought to regulate astrogliosis through the production of IL-1 (Giulian et al., 1986; Lindholm et al., 1992). This *in vitro* evidence has been supported by *in situ* localization studies that suggest the rapid and concurrent appearance of OX-42-IR microglia and cells immunolabeled with IL-1 is consistent with the idea

that reactive microglia produce IL-1 *in vivo* in response to transection of CNS tracts (Fagan and Gage, 1990).

In the studies of this thesis, neither the microglia-impregnated nor the control tubes ever demonstrated the presence of GFAP-IR cells, even in the regions of inflammation immediately surrounding the tube walls. This was especially evident with the degrading polymeric implants that were surrounded and infiltrated by microglia/brain macrophages, although the infiltrates were comprised of other elements, many of which were laminin-IR elements and peripheral Schwann cells.

Recently, Windle's experiments were repeated in the rat model using a traumatized (crushed) spinal cord paradigm (Guth et al., 1994). Compared to control animals, which received daily vehicle injections intraperitoneally, treatment with LPS resulted in significant reduction of cavitation and increased the number of cells and axons in the lesion. They were well vascularized and filled with dense bands of longitudinally disposed, spindle-shaped cells whose origin could be traced to the central canal or to the adjacent gray matter of the spinal cord. In addition, transversely oriented bands were found to originate from dorsal roots. The cellular profiles described in these regions resemble those seen in many of the microglia-impregnated and LPS-stimulated implants, as well as some control tubes that demonstrated heavy cellular infiltration.

Unlike Schwann cell-seeded implants that promote regeneration in the injured spinal cord (Xu et al., 1995), semipermeable guidance channels filled with only collagen- or laminin-containing gels without cells do not induce regeneration in peripheral nerves or late developing spinal tracts (Joosten and Bregman, 1993; Valentini et al., 1987). While grafted astrocytes seeded in similar semipermeable guidance channels alone inhibit peripheral nerve

regeneration, Schwann cells are able to partially overcome this inhibition if they are provided in a sufficient, dose-dependent fashion (Guenard et al., 1994). However, antagonists of IL-1 receptors impede such peripheral regeneration accompanied by vascularization, suggesting that macrophages that produce IL-1 play an essential role in controlling peripheral nerve regeneration and angiogenesis through the release of stimulatory and/or inhibitory molecules (Guenard et al., 1991). In light of the results of this thesis, seeding microglia with Schwann cells would directly confirm whether they counteract the positive influences that Schwann cells have on the regenerative capabilities of mammalian CNS neurons.

Macrophage activities include removal of dead tissue and debris through phagocytosis, lipid recycling, and secretion of a wide spectrum of cytokines possessing trophic, mitogenic, and chemotactic properties. These activities affect the behavior of resident cells in the vicinity of the wound. It has been suggested that microglia/brain macrophages permit or support, rather than inhibit sprouting of injured axons (Battisti et al., 1995; Blaugrund et al., 1992) and that regenerative failure in the mammalian CNS may be attributed to the temporal and/or spatial deficiency of brain macrophages in the injured central fiber tracts (Perry et al., 1987; Stichel & Muller, 1994). This supports the hypothesis that the failure of CNS neurons to regenerate is, at least partly, due to an inadequate inflammatory response (Lotan and Schwartz, 1994). Micro-lesion studies further suggest that microglia/brain macrophages contribute actively to neurite growth and regeneration in the injured CNS (Li and Raisman, 1994; Chamak et al., 1994), the latter study demonstrating lesion-induced expression of the ECM molecule thrombospondin (TSP) on brain macrophages in regions of neuritic sprouting. This laminin-like glycoprotein involved in processes such as

platlet aggregation (Lawler, 1986) has been shown to promote the adhesion and neuritic outgrowth from both central and peripheral neurons in culture (O'Shea et al., 1991; Osterhout et al., 1992)

Investigations into the association of microglia with axonal growth in the injured CNS suggest that microglia show rapid and dramatic responses to injury, and to the deposition of artificial fiber tracts made by Schwann cells (Raisman et al., 1993). These studies demonstrated that cultured Schwann cells injected into the adult CNS created a tract along which they became elongated parallel to the axis. Within the Schwann cell domains microglia also took up an elongated form and thus in parallel to the Schwann cell processes and axons. Schwann cell differentiation and function is thought to require the interaction not only with axons but also with ECM substances and some secretory products of fibroblasts (Bunge, 1983). Unless the CNS tissue is histologically altered in some way by injury or disease, the Schwann cell does not find the simultaneous contact with both axon and ECM that it needs for an important intermediate step in its normal differentiation. Schwann cell function within CNS tissues may depend on connective tissue changes as well as the ability of the Schwann cell to invade the CNS.

Studies of microglia *in vivo* after peripheral axotomy suggest that microglial activation is related to the repair process of peripheral nerves and that they may have a neuroprotective effect on damaged, but viable, motoneurons that successfully regenerate (Streit & Kreutzberg, 1988). The biological significance of microglial hyperplasia in the environs of axotomized motoneurons is unknown. Speculations still exist as to whether microglia are activated by local signals secreted by the damaged neurons or whether some form of activating signal is retrogradely transported from the periphery (Streit and Graeber, 1993).

An interesting correlate to these studies is that axotomy of central tracts (vs. peripheral nerves) does not activate microglia in the projection nuclei which undergo slowly progressive retrograde atrophy (Barron et al., 1990). The absence of proliferative, reactive microglia in these nuclei may correlate with the inability of the CNS to regenerate central processes. In addition, the ECM molecule thrombospondin (TSP), which is found in neural systems of species capable of regeneration, is also present in murine spinal motoneurons and their axons, while no TSP is found in the surrounding neuropil or in association with glia (Hoffman et al., 1994).

In this regard, mounted cryosections from some control and microglia-impreganted PTFE implants were sent to Dr. K. Sue O'Shea at University of Michigan who has a polyclonal antibody against TSP recognized in every species she has studied. She agreed to stain the sections with her antibody, along with OX-42 antibody which I provided. The results indicate that there is expression of TSP throughout the microglia-impregnated implants but restricted to the tube walls in control implants. This lends to entirely separate avenues of interpretation, but it does support the hypothesis that transplanted, and perhaps endogenous, activated microglia have neurotrophic properties through the production of ECM molecules.

Future Directions

Future experimentation is clearly warranted and several changes in protocol are recommended. The first would be to modify the PTFE tubes to create thinner walls (~100μm) having less porosity (<5μm) in order to minimize displacement of the spinal cord once implanted, as well as to prevent the infiltration of cells via the walls. Another alternative would be

to replace them with PAN/PVC polymeric tubes used in other intraspinal transplantation studies (Xu et al., 1995).

Because the structural, chemical and biological aspects of the matrix design may all play a role in determining the fate of regenerating axons, matrices in future experiments should be more accessible to cells during the seeding procedure. An example of such a matrix would be Matrigel™. However, because it already contains several ECM components and quickly degrades in the host tissue, biosynthetic hydrogel matrices with biologically relevent peptides covalently bound to the polymer backbone may be a promising consideration for future studies (Bellamkonda and Aebischer, 1994).

In situ hybridization studies are the next logical approach in determining what cytokines/growth factors, or potentially neurotoxic molecules are secreted by microglial cells. Isolating a unique protein expressed by microglia not shared by peripheral macrophages will, of course, be the next big advance in understanding the mechanisms for microglial activation, as well as differentiating grafted microglia from their host counterparts. In this regard, experiments involving antisense probes to "knockout" putative genes encoding for cytokine/growth factors expressed by grafted microglia (i.e., TGF-β, IL-1, NGF) may elucidate the mechanisms by which these cells produce their effects in the injured CNS. In addition, microglia derived from homozygous mice that are deficient in the production of a particular protein (i.e., TGF-β-) can be transplanted into the CNS of a strain encoding the antigen to determine whether trophic effects are due to grafted versus host microglia.

Lastly, a clinically more relevent and applicable approach to assess the effects of microglia in the injured CNS is to inject them directly into

experimental lesions, as has been done with macrophages in peripheral wounds (Danon et al., 1989). In addition, other approaches may be taken in future transplantation experiments with neural suspension grafts, such as to enrich them with syngeneic, cultured microglia in an attempt to promote increased vascularization and neuritic growth into grafts by the host.

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BIOGRAPHICAL SKETCH

Alexander George Rabchevsky was born on April 10, 1966 in Washington, D.C., and his Russian parents , George and Olga, gave him the nickname "Sasha." Always mischevious and ever athletic throughout his career at Albert Einstein Senior High School in Maryland, Sasha went on to play varsity football at Hampden-Sydney College, Virginia, in 1983, and to study as well. In the summer of 1985, however, his interest in Neuroscience was tragically inspired during his recovery from a motorcycle accident. It left him paralyzed from the chest down due to spinal cord damage precipitated by fractured T5 and T6 vertebrae. After taking correspondence courses during rehabilitation, he returned to Hampden-Sydney a year later and assumed a heavy course load in the sciences to graduate with a B.S in Biology in the Fall of 1987, only a semester behind his original graduating class.

Shortly after graduation, Sasha obtained a position as a biological technician for Dr. Cinda J. Helke in the Pharmacology Department of the Uniformed Services University of the Health Sciences (USUHS), Bethesda. His studies focused on the connectivity and neurochemical content of bulbospinal projections and visceral afferent neurons. While there, he learned of Dr. Paul J. Reier's work at the University of Florida and contacted him regarding a graduate position. For the past five years, his laboratory has served as Sasha's home away from home.

After a year in the lab, Sasha was granted a two-year studentship award from the Rick Hansen Man in Motion Legacy Fund in Canada for his project

entitled, "Studies of Kinesin in Normal and Axotomized Central Neurons." He then became interested in microglial cell involvement in the neuroimmunology of neural transplant rejection, and their potential role in CNS regeneration during inflammation. As his thesis proposal developed, Sasha helped with the construct of, and provided the preliminary data for, a grant awarded to Dr. Streit entitled, "Depletion of Microglia from Neural Allografts: A New Approach Toward Reducing Graft Rejection," awarded in July 1994. However, the main efforts of his thesis have, instead, focused on the characterization of microglial transplants into the injured rat spinal cord.

Outside of the laboratory Sasha met his fiancee, Gisele Catherine Legare, in the summer of 1992, and they are to be married on June 3 of this year before they embark on a new life in a new country, France. Sasha was offered, and he accepted, a postdoctoral position in the Neuroplasticity research laboratories of Marc Peschanski, M.D./Ph.D., part of the National Institute of Health and Medical Research in Creteil, France, fifteen minutes southeast of Paris. His work there will involve mastering the French language, and developing techniques using transfected monocytes to deliver protective substances (i.e., CNTF, NGF) to reactive lesions in animal models of stroke and EAE, where macrophages accumulate.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Paul J. Reier, Chairman
Mark F. Overstreet Professor of
Neurological Surgery and

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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